THE BIOCHEMICAL JOURNAL

CAMBRIDGE UNIVERSITY PRESS
LONDON: FETTER LANE, E.C.4

H. K. LEWIS AND CO., LTD., 136, GOWER STREET, LONDON, W.C. 1

WHELDON AND WESLEY, LTD., 2–4, ARTHUR STREET, NEW OXFORD STREET, LONDON, W.C. 2

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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

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VOLUME XXVIII 1934

PART I, pp. 1 to 1148

CAMBRIDGE
AT THE UNIVERSITY PRESS
1934

PRINTED IN GREAT BRITAIN

Vol. XXVIII, No. 1 1934

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Fetter Lane, E.C. 4
also
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan
TOKYO: Maruzen Company, Ltd.

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1934

Price Fifteen Shillings net

PRINTED IN GREAT BRITAIN

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I. THE DISTRIBUTION AND ORIGIN OF SULPHUR IN WOOL.

I. METHIONINE IN WOOL.

By JOHN BARRITT.

From the Wool Industries Research Association, Torridon, Headingley, Leeds.

(*Received November 9th, 1933.*)

THE distribution of sulphur in protein has long been a subject of interest, and the work of Osborne and numerous other investigators on the sulphur in vegetable proteins indicated the probable presence of at least two forms of organic sulphur; further, Dakin [1920] in his amino-acid analysis of gelatin found sulphur compounds other than cystine. The isolation of methionine [Mueller, 1921] and its synthesis [Barger and Coyne, 1928] clearly established the existence of at least one other sulphur-containing amino-acid. Pirie [1932] has isolated methionine from caseinogen in 1 % yield, corresponding to approximately 50 % of the total sulphur; generally it may be said that this amino-acid appears to enter into the composition of proteins to a considerable degree and may have far-reaching effects in nutritional work.

Scleroproteins, particularly wool and hair, contain a large proportion of cystine which accounts for as much as 70 % of the total sulphur [Barritt, 1927].

Subsequently Marston [1928], Rimington [1929, 1, 2] and Barritt and Rimington [1931], using colorimetric methods devised by Sullivan [1926] and Folin and Marenzi [1929], were able to show that substantially all the sulphur in wool and hair could be accounted for as cystine, though it must be noted that 0·2 % of methionine has been isolated from wool [Mueller, 1923].

The possible non-specificity of the Folin-Marenzi reagent for cystine has been discussed, and the recent experiments of Jones and Gersdorff [1933] on the rate of liberation of amino-acids during the hydrolysis of caseinogen are of interest in that they indicate sources of possible error in the determination. A rapid initial increase in the depth of colour developed, the maximum being reached after 45 minutes corresponding to 0·53 % cystine, then an abrupt drop occurred and finally a gradual falling away to a constant value of 0·33 % after 18 hours. Using the Sullivan [1926] reagent, the cystine rose steadily to a constant value of 0·33 % after 6 hours. It appears that a reasonable time for the hydrolysis is necessary when using the Folin-Marenzi technique.

Isolation methods for the quantitative estimation of methionine are difficult and much loss of material occurs.

Baernstein [1932, 1] has developed a technique for the estimation of methionine in proteins based on methods described by Kirpal and Buhn [1915] and Pollack and Spitzer [1922] for the determination of methylthiol groups. In this method methyl iodide liberated by digesting the protein with hydriodic acid is estimated by passing into alcoholic silver nitrate and determining the silver iodide. Baernstein assumes that this volatile iodide arises solely from the methylthiol group of the methionine, the assumption being supported by the

facts that no methoxyl or *N*-methyl group has been shown to occur in proteins, no other compound containing a methylthiol or similar group has been isolated and lastly that the amino-acids other than methionine do not liberate volatile iodide when treated with hydriodic acid. Such determinations together with those of sulphydryl and disulphide sulphur have enabled Baernstein [1932, 2] to account for the total sulphur in a large number of proteins, though keratins were not studied. In many instances the methionine content is high and with caseinogen methionine-sulphur accounts for 84 % of the total sulphur. The validity of the assumption that methionine is the sole source of volatile iodide may be questionable, but such determinations are not without significance in that they appear to give at least an upper limit for methionine content.

A series of determinations of methionine on various types of wool was made, a detailed account of the modifications found necessary being given below.

Table I.

	Quality*	Total sulphur on dry wt. %	Methionine on dry wt. %	% of total sulphur as methionine-sulphur
Welsh Mountain S. 64	58s	3·97†	0·44	2·4
Welsh Mountain Kemp	Very coarse	3·29	0·64	4·1
Romney Corriedale	36s	2·96†	0·66	4·8
Camel Hair	80s	3·37	0·67	4·3
Scotch Blackface (fine)	56s	3·73	0·57	3·2
Scotch Blackface (coarse)	Very coarse	3·10	0·51	3·4
Alpaca	60s	3·68	0·54	3·2
Mohair	36s	3·20	0·63	4·3
Lincoln	36s	3·46	0·50	3·0
Western Australian	70s	3·73	0·55	3·2
New Zealand	36s	3·40	0·50	3·2

* The qualities of the samples were determined by comparison with standard wools, and the author is indebted to Miss A. L. Walker for these determinations. Qualities of 80s, 70s, 60s, 56s and 36s correspond to diameters of 1·92, 2·07, 2·24, 2·83 and $3{\cdot}90 \times 10^{-3}$ cm. respectively.

† Indicates sulphur by the Carius method, others by the Benedict-Denis.

Experimental.

Preparation of samples. The wool samples were removed from the fleece in locks, which were opened out and degreased by washing in three baths of benzene at 60°. Small particles of foreign matter were removed by hand-sorting and the wools given a final treatment with benzene. The samples were dried at room temperature and well washed in six changes of distilled water, air-dried, and allowed to attain a uniform moisture content. Samples for methionine, sulphur and moisture content determinations were weighed off. The dry weights of individual samples were obtained by reference to determinations of moisture content made on samples taken at the beginning and end of the sampling. The moisture determination on the reference sample was carried out by passing a stream of dry air through the wool contained in a special bottle, maintained at 106° in an electrically heated oven, the procedure being fully described in an earlier paper [Barritt and King, 1926].

Determination of methionine. The technique employed was substantially that due to Baernstein [1932, 1] though with wool minor modifications were necessary.

Using a single washing-tube containing cadmium sulphate and a suspension of red phosphorus, it was found that a little hydrogen sulphide escaped and passed over into the alcoholic silver nitrate. This difficulty probably arises because of the very high cystine content of the wool-keratin and was overcome

by inserting a small tube containing glass wool or paper pulp moistened with cadmium sulphate solution between the washing-tube and the first silver nitrate tube. As a further check on the efficiency of the removal of hydrogen sulphide, a cadmium sulphate test-paper was inserted immediately before the first silver nitrate tube; if any signs of yellowing of the paper occurred, the experiment was abandoned.

In order to prevent the boiling hydriodic acid sucking up the side-tube and into the sulphuric acid wash-bottle, a bulb 2–3 cm. in diameter was blown on the side-tube; it was found to be quite effective. The duration of the run was 7–8 hours, this being sufficient in the case of wool-keratin.

Alcoholic silver nitrate (10 ml. of the order of $N/10$) was distributed between the two absorption-tubes. At the end of the run the tubes were washed out into a small beaker and the liquid evaporated on the steam-bath to about 10 ml. and made up to 50 ml. The contents of the flask were filtered through a dry paper and 20 ml. aliquots titrated with potassium thiocyanate (order of $N/50$), 0·5 ml. ferric alum indicator being added. The amount of volatile iodide was readily obtained from these data.

The blank on the apparatus was calculated from the results of eight determinations on each wool, four using 0·5 g. and four using 1 g. samples, these being taken alternately. A typical run is shown in detail as follows:

Welsh Mountain Wool. Sheep S. 64.

No. of exp. ...	1	2	3	4	5	6	7	8
Wt. of wool taken (g.)	0·5867	1·0658	0·5154	0·9535	0·5342	0·9467	0·4770	1·0640
ml. of 0·01933 KCNS equivalent to AgI	1·40	1·91	1·28	1·97	1·21	1·76	1·15	1·99

Moisture: 0·9698 g. of conditioned wool on drying out lost 0·1194 g.

Combining the above results, the blank on the run corresponds to 0·55 ml. and the methionine content of the wool to 1·35 ml. KCNS per g., giving 0·44 % methionine on the dry weight of the wool.

The value of the blank so obtained was found to agree well with values obtained when no wool was added. Although the blank is high in relation to the amount of KCNS corresponding to the methionine it is felt that the method employed is satisfactory in determining with reasonable accuracy the methyl iodide produced from wool-keratin.

Determination of sulphur content of wool. This was determined either by the Carius method using samples of 0·5 g. [Barritt and King, 1926] or by the method of Benedict [1909] and Denis [1910].

Using the latter method a sample of 0·8 to 1 g. was hydrolysed for 2 to 4 hours with 20 ml. 6N hydrochloric acid, the hydrolysate being made up to 50 ml. Aliquots of 20 ml. were taken and the determination then proceeded with as described by Rimington [1930]. This method is somewhat inaccurate owing to the fact that on hydrolysis, particularly in the early stages, small quantities of hydrogen sulphide are liberated. It is hoped to deal more fully with the determination of total sulphur in keratins in a separate paper, but in view of the work of Gortner [1922], which shows that no appreciable decomposition of cystine occurs during the time necessary for a protein hydrolysis, it is suggested that this liberated hydrogen sulphide arises from the methionine or some unknown sulphur compound of wool-keratin.

Discussion.

The results given in the above table for a fairly representative series of wools do not justify any rigorous correlation between the methionine content and the type of wool, as was possible with the total sulphur in wools, where it was shown [Barritt and King, 1926] that total sulphur is higher in the finer wools grown under similar conditions. The sample of camel hair is of interest in that only 94 % of its total sulphur could be accounted for as cystine-sulphur [Rimington, 1929, 2] but its methionine content is not abnormally high.

The methionine content of the wools examined lies between 0·44 and 0·67 % of the dry weight, the methionine-sulphur varying from 2·4 to 4·8 % of the total sulphur. This quantity of methionine-sulphur accounts for about 3–4 % of the total sulphur in wool and does not seriously affect the previous conclusion that sulphur in wool occurs almost wholly in the form of cystine, though the possibility of undiscovered sulphur-containing amino-acids must not be overlooked. A possible explanation for the somewhat high values recorded for cystine-sulphur in keratin using the Folin-Marenzi technique is afforded by the work of Butz and du Vigneaud [1932] on the hydrolysis of methionine. On hydrolysis with acids, especially sulphuric acid, methionine gives homocystine, the next higher homologue of cystine, which is reactive to the Folin-Marenzi reagent; thus it appears possible that some methionine-sulphur may have been returned as cystine-sulphur using the colorimetric method of Folin-Marenzi.

The actual synthesis of wool-protein by the sheep is somewhat outside the scope of the present work, but it is interesting to note that the form in which the sulphur is obtained has been the subject of much speculation. Many calculations have been made of the necessary cystine content of herbage [Rimington and Bekker, 1932; Henrici, 1932; King, 1933; Woodman and Evans, 1932; Robertson, 1928] based on the assumption that cystine as such is essential for ultimate wool synthesis. These calculated values vary widely, some falling within, others outside the values for cystine in grasses as determined by Aitken [1930] and Evans [1931].

In determining cystine values obtained on a grass hydrolysate, the probability of loss of cystine due to interaction with carbohydrate material must be considered, and recently Lugg [1933] has shown that considerable loss of cystine occurs when it is hydrolysed by hydrochloric acid in the presence of sucrose and glycine. Preliminary determinations (by the author) of cystine in straw and potatoes show very high values, using the Folin-Marenzi technique, but only small amounts with the Sullivan reagent, illustrating the difficulties which occur when cystine is directly determined in complex materials.

Assuming a deficiency of cystine in the herbage, Rimington and Bekker [1932] have suggested that inorganic sulphur is elaborated by micro-organisms of the intestinal tract into bacterial protein which is then absorbed and is available for cystine synthesis, some support being given to this hypothesis by the work of Voltz [1920].

The total organic sulphur in herbage [Aitken, 1930; Evans, 1931] is however well above the sulphur requirements of wool production, but its actual distribution is as yet undetermined, though the work of Miller and Chibnall [1932] suggests the presence of methionine. In view of the possible replacement of cystine by methionine in growth experiments [Weichselbaum *et al.*, 1932; Jackson and Block, 1932], the suggestion of Jackson and Block, that cystine and methionine form a freely interconvertible system of which only one member is indispensable to a limited degree, and that an adequate supplement

of the other may suffice to provide a common metabolite of the two, is of especial interest in a study of wool production, though their experimental data do not eliminate the possibility of alternative explanations.

SUMMARY.

1. The methionine content of various wools has been determined. It is shown that the amount present does not substantially affect previous work on the relation between total and cystine-sulphur in wool, which indicated that substantially all the sulphur in wool could be accounted for as cystine.

2. The suggestion is made that methionine occurs widely in feeding stuffs and grasses and may play an important rôle in the ultimate synthesis of wool- and hair-proteins.

The author wishes to express his thanks to the Council of the Wool Industries Research Association for permission to publish this account, and to Prof. A. T. King, formerly Head of the Chemistry Department, for the interest he has taken in the work. Throughout the course of the investigation, the author's assistant Mr F. F. Elsworth, has given valuable help.

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II. INOSITOL METABOLISM IN THE MAMMALIAN HEART.

By LEWIS BLAND WINTER.

From the Physiology Department, University of Manchester.

(*Received November 11th, 1933.*)

The significance of the occurrence of inositol in animal tissues is still unexplained, and it is a matter of dispute whether inositol is metabolised by animals. Rosenberger [1910] asserted that the inositol content of muscle was increased during autolysis but gave no figures. Needham [1923] compared the inositol contents of different tissues of a freshly killed rabbit and of one killed two days before: he remarks "It is certainly interesting that the muscle from the freshly killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger that tissues contain an 'Inositogen,' though his conclusions were drawn from the comparison of fresh rabbit muscle and commercial beef; tissues perhaps hardly comparable."

Boyland [1928], on the other hand, records three experiments with pig's heart and as a result suggests that inositol decreases during survival changes in muscle and is a precursor of lactic acid. For a review of the literature see Needham [1926].

Experimental.

Work on inositol has been hampered by lack of a convenient method for its estimation. Needham [1923] made use of acetone for extraction of the tissues, and precipitated the inositol with basic lead acetate: the inositol was then thrown down with strong alcohol and the carbon in the precipitate was determined. Boyland [1928] used a modification of Needham's method, the modification consisting in a direct weighing of the precipitate in place of a micro-estimation of carbon. Boyland remarks that "The method is probably not very specific, but a more reliable method could not be found." Complete extraction of even minced tissue is difficult unless large quantities of solvent are used, and then it is not easy to make the process quantitative: in addition, in the present work it was desirable to use a method by which cell enzymes could be destroyed or inactivated as soon as possible after removal of the tissue from the body. It was decided to take advantage of the stability of inositol to hot alkali. Rosenberger [1910] made use of boiling KOH solution for the estimation of inositol, but his drastic treatment has been criticised by Momose [1916]. The principle of the method employed in the present work is as follows. The tissue (30–40 g.) is disintegrated by heating with an equal volume of 10 % aqueous KOH: after neutralisation and cooling, a large amount of impurity is removed with normal lead acetate: the inositol is precipitated by basic lead acetate, and after being freed from the lead compound is precipitated by baryta in alcoholic solution. After decomposition of the barium precipitate the inositol is dried, recrystallised and weighed. The fresh heart-muscle of dogs was used throughout. The details are best described in the form of a typical experiment.

The animal is killed by severing the carotid arteries; the chest is immediately opened and the heart removed[1]. Excess of fat is removed with a few strokes of the knife, the heart is divided roughly into two, one-half is immediately weighed and then cut up over a flask containing 10 % KOH at 100°; when all the pieces have been dropped into the potash a condenser is fitted and boiling is continued for the required period. The amount of KOH solution should be about 1 ml. for each gram of tissue. Meanwhile the other half of the heart is allowed to undergo survival changes, then the muscle is cut up into potash and the procedure is the same as for the first half. After the boiling with alkali is completed the fluid is made acid to litmus with glacial acetic acid, cooled to about 50°, and a rapid current of hydrogen is passed through for an hour to remove hydrogen sulphide liberated by the action of the potash on sulphur compounds in the muscle. Half the volume of water is added, and after standing for a day in the cold chamber the fluid will filter clear. To the filtrate is added a saturated solution of normal lead acetate, 0·2 ml. per g. of muscle, and a day is allowed for complete precipitation.

After centrifuging (the precipitate being discarded without washing) ammonia is added to the clear fluid until further additions give no precipitate. The precipitate is very bulky, but may be collected into two centrifuge-tubes of 25 ml. capacity if a very high speed machine such as the "Leune," made by Messrs Hearson, is used. To the clear liquid lead acetate (0·2 ml. per g. of tissue) is added, followed by ammonia. The precipitate is collected in the same cups as the first. A further precipitation, using half the quantity of lead, is performed. The combined precipitate is twice washed very carefully with 75 % ethyl alcohol, and the cups with the precipitate are then dried *in vacuo* over sulphuric acid. The precipitate is then weighed and collected in one centrifuge-cup; it is ground up with 2*N* sulphuric acid and about 5 ml. of water until the mixture is acid to litmus. About 2 ml. of acid are required for each g. of the lead compound. The mixture is centrifuged and the liquid is measured and poured into a 100 ml. flask. The precipitate is stirred up with 5 ml. of water, and sufficient acid (about 0·3 ml.) is added to make the reaction acid. The process is repeated. To the combined extracts in the flask 3 volumes of strong alcohol are added. Precipitation takes several days. The fluid is centrifuged, and the precipitate is discarded without washing. An excess of 10 % baryta in methyl alcohol is added, and after 10 minutes the liquid is centrifuged and the precipitate washed with 75 % alcohol, and suspended in 100 ml. of 75 % alcohol in a tall cylinder. Care must be taken to obtain a uniform suspension, and this is best effected by first stirring up the precipitate with very small amounts of alcohol. The barium-inositol compound is decomposed by passing in a stream of CO_2 at 40° for 2 hours. Two days are allowed for precipitation of carbonate, the fluid is centrifuged and concentrated to dryness under reduced pressure. The residue is washed with dry methyl alcohol, taken up in 5 ml. water and allowed to evaporate slowly over sulphuric acid. Usually the inositol crystallises but occasionally is left as a glass. Recrystallisation is effected by dissolving the solid in 2·5 ml. water and adding 20 ml. strong alcohol. After 2 weeks the crystals are washed with methyl alcohol, dried and weighed. The melting-points of different preparations made in this way varied between 211 and 215°. Two preparations taken at random gave the following analytical figures (micro-analyses Schoeller) C 40·36, H 6·79 %; C 40·44, H 6·75 %; $C_6H_{12}O_6$ requires C 40·00, H 6·66 %.

[1] Dr T. H. B. Bedford removed all the hearts, and I am grateful for the rapidity and skill which he showed in all these experiments.

To determine whether survival changes in heart muscle affected the inositol content, one half heart was immediately treated with boiling potash, while the other was kept in an atmosphere of nitrogen for 90 minutes at 37°. Each was boiled with potash for 30 minutes. The following four experiments show that the inositol content is increased on incubation of the tissue.

Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
22·3	29·5
10·0	40·0
30·2	61·6
10·0	23·7

In the following series the time of boiling was increased, to determine whether inositol was being set free from some combination by the action of the alkali.

	Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
Boiled for 1 hour	7·8	8·3
	54·1	54·0
	9·2	79·3
	40·5	45·0
Boiled for 2 hours	43·9	68·3
	64·9	74·5
	120·0	128·0
	122·0	166·0
Boiled for 5 hours	79·0	114·0
	112·3	122·0
	147·0	185·0

Two facts emerge from this series: the amount of inositol increases with the time of action of the alkali, and the survival changes still cause more inositol to be formed than in the corresponding half heart killed immediately. To decide whether 5 hours' boiling with 10 % KOH is sufficient to liberate the whole of the "combined" inositol, hearts were divided into two, and one half was boiled for 5 hours, the other for 12; each half was thrown into potash immediately after weighing.

5 hours' boiling mg. inositol per 100 g.	12 hours' boiling mg. inositol per 100 g.
108·3	94·0
40·2	34·4
61·7	61·0
45·7	59·5

It is evident that the maximum liberation of inositol by the potash is effected in 5 hours, within the limits of experimental error of the method, necessarily large. Four experiments were performed to estimate this error, the heart being divided into two equal pieces and the inositol estimated after boiling for 5 hours.

mg. inositol per 100 g.		Percentage error	Average error
108·8	101·2	7·5	
119·0	106·2	12·1	±13 %
68·7	53·0	29·6	
78·9	75·3	4·8	

That the maximum yield of inositol from heart muscle is found after survival changes have taken place is confirmed by the following series of experiments, in which the hearts were divided into three approximately equal parts, A—killed

at once and boiled 30 minutes, B—killed at once and boiled 5 hours, C—incubated 90 minutes in nitrogen at 37° and boiled 5 hours.

Muscle killed before survival changes		After survival changes
A. Boiled ½ hour	B. Boiled 5 hours	C. Boiled 5 hours
mg. inositol per 100 g. tissue.		
59·7	83·0	102·0
12·8	18·6	36·8
49·5	71·0	102·0
83·0	112·0	141·0

In individual hearts, the value of A is that of the "free" with a small amount of the "combined" inositol, for it must be assumed that boiling for even half an hour with potash liberates some inositol from combination: the difference between A and B gives the value for the "combined" inositol (too low, for the reason already mentioned), and the difference between B and C is the amount of inositol produced during survival changes in the muscle; this will be referred to as the "new-formed" inositol. All the above experiments were carried out with a minimum of cell destruction. To obtain further light on the mechanism of production of the "new-formed" inositol, it was desirable to determine whether moderate cell disintegration, as in mincing, interferes appreciably with the production of the "new-formed" inositol. The hearts were minced and divided into two nearly equal parts. One, after weighing, was placed at once in twice the usual volume of boiling potash (10 % in 0·9 % sodium chloride) and the other was incubated in 0·9 % saline (1 ml. for each g. tissue) for 90 minutes at 37° in an atmosphere of nitrogen. At the end of this time 20 % potash in amount equal to the original volume of saline was added. Boiling was for 5 hours in each case.

Muscle killed immediately	After survival changes
mg. inositol per 100 g. tissue.	
60·0	69·5
78·6	96·6
87·6	95·0
78·4	97·0

It is evident that new formation of inositol has taken place. The possible effect of phosphate buffers of different p_H was then tried. The hearts were minced and divided as nearly as possible into three equal parts. Column A refers to the tissue which was placed at once in 10 % potash. In column B are the figures for the tissue incubated for 1½ hours in buffer p_H 5, in column C those for p_H 8·0. All were boiled for 5 hours with the same volume and concentration of alkali.

A	B	C
mg. inositol per 100 g. tissue.		
72·0	66·0	66·0
44·0	73·0	65·7
58·7	61·6	71·5
62·9	92·0	87·0

In three of the experiments the yield of inositol was increased on incubation of the muscle, but there was no significant difference between the acid and alkaline media.

In the following series of experiments a known amount of inositol was added to one half heart, and the whole extraction process was carried through to estimate how much of the substance is lost in the method of extraction and to obtain approximate figures for the free, combined, and "new-formed" inositol in the dog's heart. The average recovery was 73·2 %.

mg. inositol added	mg. inositol in each half heart		Difference	Percentage recovered
71·9	28·4	80·0	51·6	71·8
76·0	13·6	65·5	51·9	68·1
85·0	16·2	78·6	62·4	73·5
79·2	15·5	78·8	63·2	79·3

Discussion.

Though the experimental error of the method is considerable, it has the advantage of certainty that inositol is being estimated, and it seems safe to conclude that in the heart muscle of the dog the inositol is of three kinds. Firstly, that lying free in the muscle: it is difficult to say what proportion this bears to the whole, for survival changes were occurring between the death of the animal and the killing of the muscle cells in the boiling potash, and there is also the hydrolytic action of the alkali to be considered during the 30 minutes' boiling. It may be that there is no free inositol in muscle, and if steps were taken to reduce survival changes to a minimum, a very small value might be obtained. Secondly, the combined inositol, that which is liberated completely after 5 hours' boiling with potash: the source of this is unknown. Thirdly, the "new-formed" inositol, that which appears during survival changes in the muscle on incubation in an atmosphere of nitrogen: the possibilities are that it is synthesised, or that it is liberated by enzymic action from some combination which is not split by boiling alkali. These problems must be left for further consideration.

Summary.

1. A method is described for the estimation of inositol in muscle.

2. Survival changes in the heart muscle of the dog are accompanied by an increase in the inositol content.

3. Evidence is brought forward for the existence of free, combined and "new-formed" inositol in the heart muscle.

It is a pleasure to thank Prof. H. S. Raper for his interest and advice throughout the work. Part of the expense was defrayed by the Government Grant Committee of the Royal Society.

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III. CHEMICAL PROPERTIES OF PUBERULIC ACID, $C_8H_6O_6$, AND A YELLOW ACID, $C_8H_4O_6$.

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(*Received November 13th, 1933.*)

The two acids formed from glucose by *Penicillium puberulum* Bainier and *P. aurantio-virens* Biourge were first isolated and investigated by Birkinshaw and Raistrick [1932]. Further investigations are recorded below.

Puberulic acid $C_8H_6O_6$.

Improved isolation of puberulic acid. The starting material, which was kindly supplied by Prof. Raistrick, consisted of a mixture of puberulic acid and the yellow acid $C_8H_4O_6$. This mixture had been obtained by crystallisation from methyl alcohol of the ethereal extract from the acidified nickel salts, as described by Birkinshaw and Raistrick [1932], and their method was followed in the preparation of well crystallised diacetylpuberulic acid. Dilution of the acetylation mixture with glacial acetic acid has proved to be useful in order to minimise charring. Diacetylpuberulic acid in alcoholic solution gives with $FeCl_3$ first a reddish brown and then a green colour. Brief hydrolysis with hot aqueous ammonia (a much milder method than the boiling with *N* sodium hydroxide used by Birkinshaw and Raistrick [1932]) yields analytically pure puberulic acid which may be recrystallised from water, and it is then obtained as small, practically colourless prisms. One can thus dispense with the final purification by means of high vacuum sublimation, which is attended by such losses as to give yields of only 10–15 %.

Diacetylpuberulic acid, triturated with a little water, is mixed with a small amount of concentrated ammonia solution and heated for about 10 mins. on the water-bath in a flask loosely corked in order to avoid too rapid loss of ammonia. After cooling and acidification by hydrochloric acid, a fine crystalline powder separates which should not show any needles of the unchanged diacetyl compound under the microscope. Yield 90 %. (Found: C, 48·40; H, 3·33 %. Calc.: C, 48·47; H, 3·05 %.) Recrystallisation from water must be carried out quickly; the substance is not very soluble in hot water; M.P. 300–305° (decomp.). (Found for recrystallised product: C, 48·2; H, 3·16 %.)

Decarboxylation of puberulic acid. Careful sublimation of puberulic acid at 290–310° under atmospheric pressure yields a crystalline product which is obtained in uniform, thin, glittering leaflets when the sublimation is repeated several times. This sublimed product may be recrystallised from absolute alcohol, M.P. 233–235°; reddish brown colour reaction with $FeCl_3$. (Found: C, 54·34; H, 4·37 %. $C_7H_6O_4$ requires: C, 54·53; H, 3·93 %.)

The only benzenemonocarboxylic acid agreeing in formula and M.P. with decarboxylated puberulic acid is 3:5-dihydroxybenzoic or α-resorcylic acid (M.P. 233°; no colour reaction with $FeCl_3$). Since the M.P. of the mixture showed a depression of 32°, these two acids are not identical.

Further evidence against puberulic acid being a benzenedicarboxylic acid. Since puberulic acid gives a distinct fluorescence when fused with resorcinol and zinc chloride (at 130–140° in an oil-bath for 10 mins.), the possibility that it might be a benzenedicarboxylic acid was again considered, although Birkinshaw and Raistrick's negative findings seemed conclusive. As a series of experiments with various aliphatic and aromatic alcohols, mono- and di-carboxylic acids showed, and as Damm and Schreiner [1882] had stated, this fluorescence does not afford a definite proof of the presence of two carboxyl groups. It was possible, however, that we were not dealing with quite the same compound as Birkinshaw and Raistrick, in view of the different conditions of hydrolysis. We therefore compared puberulic acid—prepared by the new ammonia method—with 2:4-dihydroxybenzene-1:3-dicarboxylic acid and with 4:6-dihydroxybenzene-1:3-dicarboxylic acid. We are indebted to Prof. Raistrick for specimens of these acids, the only ones deserving consideration; they are distinct from puberulic acid, which cannot therefore be a benzenedicarboxylic acid.

On the possibility of puberulic acid being a phenolic monocarboxylic acid. The alkaline hydrolysis of tetramethylpuberulic acid yields interesting information.

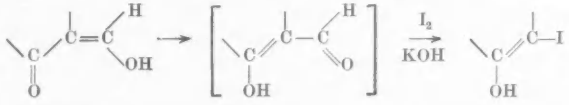
Hydrolysis at room temperature. A solution of tetramethylpuberulic acid in a measured amount of *N* NaOH was allowed to stand for 5–6 mins. at 21° and was then neutralised with *N* H_2SO_4. Long, pale yellow needles crystallised out, and after recrystallisation from water had M.P. 204° and gave no colour reaction with $FeCl_3$. They consisted of a trimethylpuberulic acid. (Found: C, 54·81; H, 5·25 %. $C_{11}H_{12}O_6$ requires: C, 54·98; H, 5·04 %.)

Hot hydrolysis, by boiling tetramethylpuberulic acid with a measured excess of *N* NaOH for 1 hour in an atmosphere of N, showed the consumption of only one equivalent of alkali on back-titration with *N* H_2SO_4. The product readily crystallised from the neutralised solution and after recrystallisation from water formed pale yellow needles similar to those of the trimethyl derivative and melting at almost the same temperature, 203°. It contained only two methoxyl groups, gave a reddish brown colour reaction with $FeCl_3$ and a M.P. depression of 25° when mixed with the trimethyl derivative. (Found: C, 52·94; H, 4·61 %. $C_{10}H_{10}O_6$ requires: C, 53·08; H, 4·46 %.) The dimethylpuberulic acid thus prepared is not identical with that described by Birkinshaw and Raistrick [1932].

A hypothetical substituted benzenemonocarboxylic acid with a tautomeric aldehyde group could explain these observations. However it was not possible to detect an aldehyde group. Attempts to isolate and characterise a Schiff's base, hydrazone, phenylhydrazone, *p*-nitrophenylhydrazone or semicarbazone were unsuccessful. The Angeli-Rimini test for aldehyde groups (reaction with benzenesulph-hydroxamic acid, followed by $FeCl_3$) was applied to puberulic acid and its derivatives and yielded sometimes positive, sometimes negative results. Since, however, puberulic acid and its derivatives can give with $FeCl_3$ alone the deep coloration, which is characteristic of aldehyde groups in the Angeli-Rimini test, the results are not conclusive.

We next tried to demonstrate the enolic nature of puberulic acid, by applying Windaus's method of detecting a tautomeric aldehyde group. This method, first

applied by Windaus [1924] to colchicine, consists of oxidising the entire tautomeric aldehyde group with iodine and potassium hydroxide.



With puberulic acid, however, the results were unsatisfactory, although, according to Windaus [1923], the method is generally applicable to *o*- and *p*-hydroxyaldehydes. Similar negative results were given by Dakin's [1909] oxidation with alkaline H_2O_2.

The stability of puberulic acid towards neutral perhydrol was very striking. When, after heating, oxidation finally took place, a complete breakdown to CO_2 was the only detectable result. During the oxidation with chromic acid, complications arose from the formation of rather stable complex salts.

The unsaturated character of puberulic acid is shown by a distinctly positive Baeyer's test with permanganate solution as well as by a yellow coloration with tetranitromethane [Werner, 1909], and on the addition of bromine in alcoholic solution. The typical $FeCl_3$ reaction of puberulic acid disappears after bromination.

Catalytic hydrogenation of diacetylpuberulic acid (in stabilised glacial acetic acid with platinum oxide, according to Adams) showed a hydrogen uptake of about $6H_2$. The isolation of the oily products of hydrogenation was difficult, largely owing to the very small amounts of starting material we possessed. Free puberulic acid seemed to be much less suitable for catalytic reduction. Gradual hydrogenation with Pd and then Pt led to an uptake of hydrogen to an indeterminate extent. More certain results were obtained from the hydrogenation of tetramethylpuberulic acid in stabilised glacial acetic acid with platinum oxide. The oily end-product gave the following analytical figures: C, 58·3, 58·44; H, 8·87, 8·67, OCH_3, 35·0, 34·92 %. $C_{12}H_{22}O_5$ requires: C, 58·55; H, 8·94; $3OCH_3$, 37·8; $4OCH_3$, 50·4 %. The figures for methoxyl deserve special attention. They represent strong evidence for a removal of an oxygen-containing group apart from a mere addition of hydrogen. The equation

$$C_{12}H_{14}O_6 + 10H \rightarrow C_{12}H_{22}O_5 + H_2O$$

must therefore include the disappearance of one methoxyl group without the loss of CH_3. The theoretical explanation is rather difficult.

The yellow acid $C_8H_4O_6$.

Attempts to transform one acid into the other have, up to now, been unsuccessful, and there is no experimental evidence for the supposition (suggested by the formula) that the yellow acid is a quinonoid body closely related to puberulic acid[1].

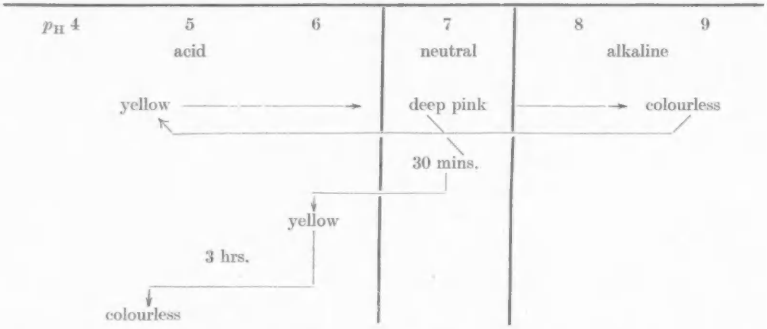
The yellow acid is not decarboxylated by the usual methods, nor can it be acetylated. Experiments for the preparation of methyl or ethyl esters yielded small amounts of crystalline products, but the analytical figures of these products did not fit with any value theoretically possible for esters of the yellow acid, although they fitted well with values for esters of puberulic acid. It thus

[1] While this note was in the press Dr H. Hoyer has obtained such evidence in my laboratory. G. B.

appeared that the yellow acid used had not been free from admixed puberulic acid. Purification of the yellow acid by recrystallisation was difficult. Only very few solvents, *e.g.* nitrobenzene or ethyl acetoacetate, were of any use.

No typical aldehyde derivatives, such as Schiff's base, phenylhydrazone or semicarbazone, could be isolated. Zinc dust distillation of the yellow product in ordinary air gave a yellow sublimate of apparently unchanged material. In an atmosphere of hydrogen however an oily substance (which later solidified) was obtained in minute yield. After purification, this product had a phenolic odour and gave a positive Baeyer's test for double bonds but no distinct $FeCl_3$ reaction; on exposure to air it became violet.

Very interesting conditions were found on neutralising the yellow acid. The yellow colour of an aqueous solution of the acid passed into a deep pink on neutralisation, and all colour disappeared in an alkaline solution. Re-acidification produced the original yellow colour but without the pink intermediate colour. The latter appeared when this re-acidified solution was again neutralised. The play of colours was reproducible several times. A neutral pink solution, on the other hand, lost all pink colour on standing for about 30 mins. and became yellow, which colour disappeared eventually after a few hours, by which time the solution had become acid. These changes are shown diagrammatically below.



Micro-titrations gave the following figures for basicity: direct titration of an aqueous solution of the acid, 1·9. Titration of a neutralised solution (calculated for a dibasic acid) which, however, reacted acid, 2·3. Back-titration of alkaline solutions, depending upon the speed of the titration itself, figures of 2·9, 3·3, 3·5.

These phenomena closely resemble those shown by pseudo-acids. If the yellow acid is quinonoid, a quinhydrone in Willstätter's sense must be considered.

SUMMARY.

(*a*) *Puberulic acid.*

1. The method of isolation has been improved.
2. The substance is a monocarboxylic acid and can be decarboxylated.
3. Various methods have failed to reveal an aldehyde group, actual or potential.

(*b*) *Yellow acid.*

1. No aldehyde group could be demonstrated; no esters or acetyl derivatives were obtained.

2. On neutralisation the acid undergoes a curious series of colour changes, behaviour suggestive of a pseudo-acid.

We are indebted to the Moray Fund of this University as well as to Geheimrat Duisberg of Leverkusen for financial help to one of us (O. D.).

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IV. FAT METABOLISM IN VITAMIN A DEFICIENCY: THE BLOOD-SERUM ESTERASE.

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(*Received November 16th, 1933.*)

The blood-serum of all animals investigated contains no true fat-splitting enzyme but contains enzymes capable of hydrolysing in greater or less degree the esters of the lower fatty acids, of which the mono- and tri-butyrins have been the esters chiefly used in experimental work.

Much knowledge has accumulated concerning the source, distribution and function of these esterases, but it is impossible to summarise it briefly for the conclusions reached are manifold and often contradictory. It has been suggested that they play a specific rôle in hydrolysing the lower fatty esters in the blood prior to their oxidation, but the esterases are widely distributed in all tissues, and therefore the most plausible hypothesis is that the serum esterases are derived from the blood and tissue cells, and as such have no specific function distinct from that of the tissue esterases generally. On this hypothesis variations in the serum esterase activity are an indication of variations in the esterase content of the tissues as a whole, or of the liver, which by its size and richness in esterase probably constitutes the main source of supply to the blood stream. The work of Kameo [1932], in which it was shown that X-ray irradiation of the liver caused a marked fall of the serum esterase content within 24 hours whereas direct irradiation of the blood had no effect, supports this theory. Observations on the tissue and serum esterases in the sarcoma-bearing rat [Green, 1934] also indicate a parallelism between the blood-serum esterase content and that of the liver, lung and kidney.

The esterase content of the serum can be affected by many factors. An increase has been observed after feeding large amounts of fat [Abderhalden and Rona, 1911; Greene and Summers, 1916], on a raw soy bean diet [Howarth and Chang, 1926], after starvation [Aguma, 1924] and after the injection of pilocarpine [Prewitt, 1925]. A decrease has been noted in conditions of severe intoxication with damage to cell metabolism [Fanconi, 1924; Caro, 1920], in lipaemia [Sakai, 1914; Fanconi, 1924], in starvation where Greene and Summers [1916] found a later rise above normal, and in the later stages of scurvy [Palladin and Normark, 1924]. Jobling and Bull [1912] found an increase in the serum esterase in immune haemolytic sera, whilst Bach [1922] found that it was independent of the type of food even when there was much fat in the diet.

The following work was undertaken primarily to determine the effect of vitamin A deficiency on the serum esterase content and the relation between this latter factor and the spontaneous infections arising in this deficiency disease.

Experimental.

The following method of estimating esterase activity was finally used after a trial of more complicated methods, since it proved efficient enough for the purpose in hand, which was a purely comparative one. Duplicate readings were reasonably close (± 2 %).

To 20 ml. of $M/80$ phosphate buffer of p_H 8·0 measured into a 100 ml. Erlenmeyer flask, 0·25 ml. phenolphthalein solution and 0·25 ml. of ester are added and the flask is brought to 37° by gentle heat; 0·5 ml. of blood-serum is then added and the flask incubated at 37° for 2 hours. The flask is then rapidly cooled and the contents are immediately titrated against $N/100$ NaOH until a pink tint appears matching that obtained with the blank control. The control flask contains no serum but is otherwise similar. In earlier experiments serum heated at 80° for 30 mins. to destroy the esterase was added to the control flask, but as this made an insignificant difference it was discontinued.

Continual shaking of the flask during the experimental period is recommended by most workers. Since it was observed, however, that this procedure produced a slight but definite diminution, varying with the rate of shaking, in the rate of hydrolysis, in the present work the flasks were not shaken. No antiseptic was added, as it was found that significant bacterial contamination did not occur over the experimental period of 2 hours.

The substrates used in the majority of experiments were ethyl butyrate and tributyrin. Similar results were obtained using ethyl acetate, phenyl acetate and triacetin as substrates, but as only a few determinations were made they are not reproduced.

Most of the experiments were carried out using young white rats from which blood was obtained by severing the vessels of the neck after previous stunning with a head blow. The blood was immediately centrifuged and the serum used within a maximum period of 6 hours. Blood was obtained from dogs by bleeding from an ear vein.

A standard basal diet [Green and Mellanby, 1928], deficient in vitamin A, was given to the experimental rats. The control rats received a supplement of some source of vitamin A, cod-liver oil (5 mg. daily), dried cabbage (0·5 g. daily) or carotene (0·02 mg. daily) being used. In some cases an ordinary stock diet was given but no significant differences in the serum esterase contents of the control animals were found whatever the source of vitamin A given. All rats received a supply of vitamin D in the form of "radiostol" (5 mg. daily).

In all cases the degree of hydrolysis, under the defined conditions, is expressed in terms of ml. $N/100$ NaOH required to neutralise the fatty acid liberated. The amount of esterase in normal rat-serum appears to vary within fairly wide limits. In 61 young rats, reared on complete diets, figures for ethyl-butyrase varying from 41 to 18 were obtained. The upper range was exceptional and the lower range was more often reached, but in the majority of cases values between 25 and 30 were obtained. The average figure for 30 control rats in the present series was 27·4 (Table II). Rats from the same family and from groups reared on the same diet and under similar conditions tended to give similar values. The serum content of tributyrinase appeared to be less variable; 10 control rats gave an average figure of 10·7 (Table II). It will be noted that rat-serum hydrolyses ethyl butyrate about three times as rapidly as tributyrin, and ethyl butyrate was therefore the substrate of choice where the amount of serum available made the use of only one substrate possible.

Criteria of vitamin A deficiency.

For the purpose of analysing the results it was necessary to formulate rough standards for the estimation of the degree of deficiency and infection in the vitamin A-deficient rat. The large individual variations which occur in rats on the same diet are well known; some die shortly after the decline period has set in whilst many survive for 2–3 weeks, the survival time depending to a great extent on the maximum weight reached before the decline commences. Neither the degree of xerophthalmia nor the severity of infection nor the loss in weight from the maximum reached is an absolute indication of the degree of deficiency. A sudden rapid fall of weight with an early death may be associated with only mild infection though the animal presumably died from rapid exhaustion of its vitamin A reserves, whilst a gradual fall in weight may be associated with a more severe infection which has had time to develop to the stage of more obvious lesions. In the former case the rat may be killed shortly before impending death and therefore at the last stage of deficiency, and in the latter case the rat killed at the same time may be in a definitely earlier stage of deficiency. All three criteria of deficiency have therefore been used, but it is obvious that no such clinical method of assessment can be exact. The results have however been averaged over many determinations, and the groupings should be reasonably accurate.

The criteria used for assessing the degrees of infection and deficiency are given below.

Criteria.

Degree of infection	
+	Small tongue abscess and/or mild infection elsewhere (*e.g.* nasal sinuses)
+ +	Large tongue abscess or other definitely infected focus (*e.g.* lung, genito-urinary tract)
+ + +	Multiple infected foci
Degree of vitamin A deficiency	
+	Very slight xerophthalmia and/or constant weight for about 7 days or slight fall in weight for a few days
+ +	Infection (+) and/or mild xerophthalmia and/or constant weight for about 14 days or gradual fall for about 7 days
+ + +	Infection (+ +) and/or advanced xerophthalmia and/or weight falling gradually for about 14 days
+ + + +	Infection (+ + +) and/or very rapid loss in weight and/or very severe xerophthalmia (panophthalmitis)

Results.

In earlier experiments the amount of hydrolysis in normal saline, instead of buffer solution, over a period of 18 hours was determined. The results obtained with 23 deficient and 17 control rats and 7 rats on a complete but quantitatively restricted diet were proportionately similar to those obtained in the later work and are not therefore given in detail.

Typical experiment. The results of one typical experiment are given in Table I in order to show the general plan of the experiments from which the average results were derived.

Table I. *Illustrative experiment.*

Days on diet	Degree of deficiency	Degree of infection	Serum esterase: Ethyl butyrate (ml. *N*/100 NaOH)	Serum esterase: Tributyrin (ml. *N*/100 NaOH)	Weight curve
I. Basal diet alone:					
27	None	None	30·0	9·7	Rising
48	+	None	18·3	8·0	Rising very slowly
55	+	None	19·8	7·5	Rising very slowly
70	+ +	+	14·1	5·4	Plateau—20 days
59	+ +	+	9·5	4·8	Falling—10 days
59	+ + +	+ +	7·2	3·9	Falling—10 days
59	+ + + +	+ + +	2·5	2·9	Falling rapidly—6 days
II. Basal and cod-liver oil (5 mg.). Food intake restricted.					
27	–	–	30·2	10·3	Rising
48	–	–	30·8	10·1	Rising
55	–	–	22·1	8·4	Plateau—7 days
55	–	+	19·9	7·2	Falling slowly—12 days
70	–	–	24·5	8·3	Falling slowly—30 days
70	–	–	23·8	8·5	Falling rapidly—10 days
III. Basal and excess cod-liver oil (3 ml.).					
27	–	–	47·9	11·3	Falling slowly—14 days
48	–	–	36·1	10·8	Rising slowly
59	–	–	42·8	10·6	Rising slowly
70	–	–	42·9	11·2	Rising slowly
IV. Basal and excess olive oil (3 ml.) and cod-liver oil (5 mg.).					
27	–	–	31·2	.	Plateau—6 days
27	–	–	28·4	.	Slight fall—4 days
48	–	–	27·0	.	Rising slowly
70	–	–	26·8	12·6	Rising slowly
V. Basal and ethyl butyrate (0·25–1·0 ml.) and cod-liver oil (5 mg.).					
27	–	–	45·0	.	Rising
27	–	+	18·3	.	Rising
48	–	–	19·4	8·0	Falling slowly
70	–	–	26·7	10·2	Falling slowly

Rats from the same family, of the same sex and of roughly similar weight were distributed as equally as possible in the different groups. In the experiment tabulated in Table I, 5 groups of rats received the following diets:

I. The basal diet, deficient in vitamin A; intake unrestricted.

II. The basal diet and cod-liver oil (5 mg. daily). Average amount of diet per rat restricted to average amount per rat consumed by rats of group I.

III. The basal diet and a relatively large amount of cod-liver oil (3 ml. daily). Intake unrestricted.

IV. The basal diet and an amount of olive oil equivalent to the cod-liver oil given to group III (3 ml.). Cod-liver oil (5 mg. daily). Intake unrestricted.

V. The basal diet and ethyl butyrate (0·25 ml. daily for 27 days, 0·5 ml. 27–48th day and 1 ml. 48–70th day). Cod-liver oil (5 mg. daily). Intake unrestricted.

The results of several experiments planned on similar lines have been averaged and the results in this one experiment are not therefore discussed further. They serve to indicate the type of individual variation in the serum esterase content met with in all experiments.

Average results. The results obtained in 47 rats at various stages of vitamin A deficiency, in 30 which had received a complete diet and in 20 which had re-

ceived a complete diet but restricted in amount to that consumed by the rats on the deficient diet, are summarised in Table II.

Table II. *Serum esterase content of rat-serum at progressive stages of vitamin A deficiency. Average results.*

		Substrate			
Degree of deficiency	No. of rats	Ethyl butyrate (ml. *N*/100 NaOH)	No. of rats	Tributyrin (ml. *N*/100 NaOH)	Diet
0	30	27·4	10	10·7	Complete. No restriction of intake
0	20	25·0	7	8·4	Complete. Intake restricted
+	10	20·4	5	7·5	Basal
+ +	10	11·6	5	4·3	Basal
+ + +	20	7·3	6	4·1	Basal
+ + + +	7	2·5	3	2·9	Basal

It will be seen that there was a progressive fall in the serum esterase content of the rat on a diet deficient in vitamin A, which commenced at the time when the rat ceased to grow and reached a very low figure near the time of spontaneous death. The rats in the last stage of deficiency (+ + + +) were almost moribund, and, as it was considered probable that animals on the verge of death from any cause would give similar figures, only a few determinations were made. Excluding this group, there was a fall in the serum esterase content from 27·4 to 7·3 when the substrate was ethyl butyrate and from 10·7 to 4·1 with tributyrin. When loss of weight was produced by a quantitative restriction of a complete diet the average serum esterase content was only slightly below the normal. In this group individual results varied more widely from the normal than is usual. In some instances very low values were obtained but in such cases evidence of infection was always found, and these values were rejected in averaging the results.

Evidence was obtained that the serum esterase content returns to normal if the yitamin A-deficient rat is given vitamin A. Small doses of cod-liver oil were given to 6 rats at the 2nd (+ +) or 3rd (+ + +) stages of deficiency, and they were killed individually at intervals of one week. Except in one case the serum esterase content showed a progressive rise, the rat killed at 6 weeks giving a value within the normal range. The exception gave a low figure (10·5) at the 5th week and was found still to have infected foci.

Vitamin A deficiency in dogs.

In a much smaller series of dogs[1] it was found that vitamin A deficiency affected the serum esterase content in a similar manner to that observed in rats.

Table III. *Serum esterase content in vitamin A-deficient dogs. Average figures.*

		Substrate	
Diet	No. of dogs	Ethyl butyrate (ml. *N*/100 NaOH)	Tributyrin (ml. *N*/100 NaOH)
Basal and vitamin A	8	6·5	23·5
Basal alone	7	3·5	10·7

[1] Prof. E. Mellanby kindly allowed me to obtain blood samples from his dogs.

As only a relatively few animals were investigated no attempt has been made to classify the degree of deficiency. When blood samples were taken all the deficient dogs showed nervous symptoms such as incoordination of gait; some had conjunctivitis but all were fairly active. The results are summarised in Table III and it will be noted that in the dog the tributyrinase content of the serum is much greater than that of ethyl butyrase. Vitamin A deficiency has obviously produced a marked fall in the serum esterase content.

Effect of vitamin B deficiency.

Though loss of weight alone did not appear to affect the serum esterase it was interesting to determine whether another vitamin deficiency disease would bring about similar changes to that observed in vitamin A deficiency. To this end rats were given a diet deficient in the vitamin B complex; a diet which consisted of the basal vitamin A-deficient diet was used from which the chief sources of the B vitamins (marmite and lemon juice) were omitted. Each rat was given cod-liver oil (10 mg. daily) as a source of vitamin A. On this diet growth ceased very rapidly, and decline in weight set in within a few days in rats weighing 70–100 g. The average serum esterase contents of two groups of rats in which loss of weight had occurred as a result of feeding this diet are given in Table IV.

Table IV. *Serum esterase content of vitamin B-deficient rats. Average results.*

Diet	No. of rats	Substrate: Ethyl butyrate (ml. *N*/100 NaOH)	Substrate: Tributyrin (ml. *N*/100 NaOH)	Weight curve
Basal + vitamin B	7	32·6	11·3	Rising
Basal alone	12	27·8	11·1	Falling—4–10 days
Basal + vitamin B	6	24·5	—	Rising
Basal alone	7	21·1	—	Falling—10–21 days

The basal diet was the vitamin B-deficient diet described in text. Vitamin B was given in the form of dried brewer's yeast (1·0 g. daily).

Under these conditions there was only a slight fall in the serum esterase content though the loss of weight was roughly similar to that observed in vitamin A-deficient rats at the 2nd (++) and 3rd (+++) stages of deficiency at which there was a decided fall in the serum esterase content.

Effect of infection.

In view of the frequency of infection in vitamin A deficiency as compared with other deficiency diseases the question arose as to how far the effects described could be due to infection itself apart from the fundamental metabolic disorder. The serum esterase content was therefore determined in a number of rats suffering from spontaneous infections or in others in which infection had been induced by inoculation with material derived from infected lesions in vitamin A-deficient rats. All these rats received ample vitamin A. The type of spontaneous infection usually observed was an acute enteritis which may occur in epidemic form in the rat, though their diet is complete. The results have been classified together, as the type or source of the infection did not appear to affect the response in the serum esterase content. It will be observed from the summarised results given in Table V that infection itself produces a distinct

fall in the serum esterase content, though the fall is not so pronounced as in the later stages of vitamin A deficiency.

Table V. *Effect of infection on serum esterase in rats on a complete diet (ethyl butyrate as substrate).*

Degree of infection	No. of rats	Serum esterase (ml. *N*/100 NaOH)
0	8	26·2
+ +	8	15·0
+ + +	5	9·9

It is obvious therefore that infection is an important factor in producing the serum changes observed in vitamin A deficiency. An analysis of the lesions found in 47 rats at various stages of vitamin A deficiency (see Table II) showed, however, that 13 of these showed no macroscopic signs of infection, and yet the average figure for the serum esterase content was 13·9. It is possible that infection sufficient to affect the serum esterase level is present before definite localisation of infection occurs, but experience with experimental infections does not suggest that a mild infection would produce the degree of change actually observed. Moreover in some rats in which rapid loss of weight had occurred and in which only slight or no signs of infection were present, a fall in the serum esterase content to very low levels was observed. It is therefore probable that the metabolic disturbance associated with vitamin A deficiency is responsible for the serum change, and that infection plays a secondary part in a greater or less degree.

Effect of excess vitamin A.

Since a deficiency of vitamin A produced a diminution of the serum esterase activity it was an obvious step to determine the effect of feeding relatively large amounts of vitamin A. Rats were therefore given the basal diet and amounts of cod-liver oil of 2 or 3 ml. daily. Control rats were given similar amounts of olive oil and a third group was given ethyl butyrate in an amount of 0·25 ml. gradually increased to 1 ml. daily. The results are summarised in Table VI.

Table VI. *The effect of supplementing a complete diet with relatively large amounts of cod-liver oil, olive oil or ethyl butyrate on the esterase content of rat-serum. Average results.*

		Substrate		
		Ethyl butyrate		Tributyrin
Supplement to complete diet	No. of rats	(ml. *N*/100 NaOH)	No. of rats	(ml. *N*/100 NaOH)
None	10	27·6	5	8·5
Cod-liver oil (2–3 ml. daily)	8	46·1	3	11·0
Olive oil (3 ml. daily)	6	28·3	—	—
Ethyl butyrate (0·25–1·0 ml. daily)	6	27·4	3	9·1

Complete diet: basal vitamin A-deficient diet + cod-liver oil (5 mg. daily).

In all of 10 rats given the large amount of cod-liver oil the capacity of the serum to hydroiyse ethyl butyrate has significantly increased. One such experiment is given in more detail in Table I and here it will be observed that there is little or no associated rise in the serum content of tributyrinase. The exact stage at which the increase occurs has not been determined; it was evidently at almost its maximum extent at the 27th day of feeding and even at the 125th day no significant change from this level was observed. The highest figure

obtained under these feeding conditions was 59·6 and the lowest 36·1; the majority gave figures between 40 and 50. No rat in the olive oil group showed this effect; the serum esterase content remained at the normal level. Only one of six rats in the ethyl butyrate group showed a rise to above normal and experiments now in progress show a tendency for a fall to occur under these conditions rather than a rise.

The rise in the serum esterase content was also observed in a few adult rats which had received excessive amounts of cod-liver oil (progressively increased up to 50 % of the diet), but where a chronic lung infection had supervened there was actually a definite fall to below normal.

No significant alteration in the serum esterase content was observed in 3 rats which received excessive doses of irradiated ergosterol (10,000 units daily) as a supplement to a normal diet for 3–5 weeks.

The rise in the serum esterase content due to feeding relatively large doses of cod-liver oil was obviously not due to an increased fat intake since a similar dose of olive oil had no effect. It is not however certain as yet whether the effect is due to excessive amounts of vitamin A. Work is in progress to determine the effects of feeding very potent vitamin A concentrates and large doses of carotene as a possible method of deciding this point.

The effect of adding vitamin A to serum *in vitro* has been determined, though a positive effect was not to be expected. Vitamin A and carotene emulsions, stable over the experimental period, were formed by adding one drop of an alcoholic solution of a very potent vitamin A concentrate or of a saturated alcoholic solution of carotene to the buffer mixture. A similar amount of alcohol was added to the control flasks. In three determinations using both normal serum and serum from vitamin A-deficient animals no increase but a slight decrease was observed in the degree of hydrolysis of ethyl butyrate. Carotene was without effect in a similar number of experiments.

Discussion.

It has been shown that vitamin A deficiency brings about a pronounced fall in the esterase content of the blood-serum. It is obvious from previous work on the subject and from my own findings that such a fall is not a change peculiar to vitamin A deficiency; amongst other causes infection and, in the rat, inoculation with sarcoma tissue [Green, 1934], have been observed. There is some reason therefore for considering it a secondary change and one unlikely to throw direct light on the physiology of vitamin A. The fact however that a similar change does not occur, except in a very much less degree, in other conditions producing loss of weight (underfeeding, vitamin B deficiency), suggests that the change may have some significance in the pathological processes due to vitamin A deficiency. Infection probably plays a part as it certainly produces a fall in the serum esterase content both in animals and in man [Green, 1934], but it has already been indicated that this is not the primary cause. Several workers have attempted to identify serum esterase with serum complement. There appears to be a parallelism between the contents of these two factors in the serum of different animals, according to Jobling and Bull [1913] but Gordon and Wormall [1929] have shown that whilst the complement and esterase contents of the serum tend to run parallel there are occasional discrepancies, and they were able to show that there was a significant amount of esterase in serum which had been heated to the extent necessary to destroy all the complement. In view however of the quantitative association of these two factors it is

interesting to compare the present results with those obtained by Osborne [1931] on the serum complement of vitamin A-deficient rats. He found a tendency towards a lowered serum complement content in vitamin A deficiency in the rat, though it is not stated at what stage of the deficiency the observations were made. It is tentatively suggested that this fall may be responsible for the lowered resistance of the vitamin A-deficient rat to infection. If there is a quantitative relationship between the serum esterase and complement it seems possible that the decrease in complement may be the result rather than the cause of infection, for the present findings show that inoculated infection produces a very definite reduction in the serum esterase content.

Future work may show that a fall in the serum esterase content occurs in many conditions in which there is a profound metabolic disturbance in the body cells. Until we know more in this connection and in regard to the function of the esterases in general, and the serum esterase in particular, it may be impossible to assess the real significance of the present findings.

Summary.

1. Vitamin A deficiency in the rat produces a large and progressive decrease in the esterase content of the blood-serum.

2. The infected rat on a complete diet also shows a considerable fall in the serum esterase content, but in rats which have lost weight on account of underfeeding or deficiency in the vitamin B complex, there is, on the average, only a very slight fall.

3. Relatively large amounts of cod-liver oil in the diet produce a rise in the serum esterase content well above normal.

4. The relation of these findings to infection in vitamin A deficiency is discussed.

My best thanks are due to Prof. E. Mellanby for his interest in this work. The author is in receipt of a personal grant from the Medical Research Council.

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V. FAT METABOLISM IN VITAMIN A DEFICIENCY: THE UTILISATION OF FAT AND THE DESATURATION OF FAT IN THE LIVER.

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(*Received November 16th, 1933.*)

THE function of vitamin A in metabolism still remains obscure. Its close natural association with the fats has led some workers to search for a possible relation to fat metabolism, but so far little of significance has been found. The profound loss of body fat in the rat that has died from vitamin A deficiency is obvious on casual examination, and it has been demonstrated by quantitative methods [Amada, 1923; Rost, 1926]. An increase in the blood-fat during vitamin A deficiency was observed by Amada [1923] and Collazo and Bosch [1923]. This loss of body fat is not superficially different however from that seen in other deficiency syndromes or in other conditions producing rapid loss of weight, such as infection and starvation. Unless the rate at which the fat disappears differs essentially from that observed in animals losing a similar amount of weight from other causes it is unlikely to be of particular significance. A more detailed survey of this loss of body fat was made by Javillier *et al.* [1929], who found in the vitamin A-deficient animal a decrease in the lipoids of the skin, liver and muscle and an increase in those of the brain and bone, whilst the spleen, lung, kidney and heart were normal in this respect. There was a decrease in the cholesterol content of all organs except the skin. They produced no evidence that these changes were specific to vitamin A deficiency.

The possibility that if vitamin A were intimately concerned with fat metabolism a large amount of fat in the diet might accelerate the onset of deficiency symptoms was explored by Drummond [1919], who found that in adult rats the addition of fat to the diet had no effect of this kind. It was shown that the adult rat on a vitamin A-deficient diet could absorb large quantities of fatty acids and effect their synthesis into fats over a period of 3 months. In view however of the now well-known fact that the adult rat may possess sufficient reserves of vitamin A in its liver to maintain it for a year or more, these early experiments cannot be considered conclusive.

The present work was designed as a preliminary attempt to investigate fat metabolism in vitamin A deficiency. Three lines of enquiry were pursued. (1) Would a large increase in the fat content of the diet produce a more rapid utilisation of the vitamin A reserves of the body? (2) Does the loss of body fat in the vitamin A-deficient animal differ quantitatively from that due to loss of weight produced by a quantitative restriction of a complete diet? (3) Is the process of fatty acid desaturation in the liver, as indicated by its content of unsaturated fatty acids, of a different order from that observed in loss of body fat due to under-feeding or infection?

The experimental results obtained in investigations covering all these points are now considered.

Experimental.

Young albino rats, raised in this laboratory, were used, and for comparative groups rats of roughly similar weight and the same sex were included in equal numbers.

Two diets were used, one a standard basal vitamin A-deficient diet [Green and Mellanby, 1928], used in this laboratory for some years, which contains roughly 8 % of fat, the other a high fat diet, containing roughly 70 % of fat, with the following composition:

Vegetable margarine	59·0 %
Olive oil	11·0
Caseinogen	18·5
Marmite	7·5
Salt mixture	4·0

The margarine was a high-class brand, consisting largely of a semi-hydrogenated vegetable fat with a small percentage of liquid vegetable oil. It was irradiated in a thin melted layer from a mercury vapour lamp at 8″ distance for 12 hours with the idea of destroying any possible trace of vitamin A. The unsaponifiable fraction was negative to the $SbCl_3$ test for vitamin A.

The olive oil was heated in a current of air at 120° for 6 hours.

The caseinogen was heated in air at 120° for 36 hours.

The McCollum salt mixture (185) was used.

In order to avoid a possible partial deficiency of vitamin B all the rats on the high fat diet received an additional supply in the form of dried brewer's yeast (0·5 g. daily). All rats used in this work received a supply of vitamin D in the form of "radiostol", 3 drops weekly[1].

The crude fatty acid content of the body was determined, after removal of the liver and alimentary tract, by saponification with alcoholic potash, conversion of the soaps into free fatty acid by treatment with sulphuric acid and extraction with light petroleum. The petroleum solution was evaporated to dryness and the fatty acids weighed.

The iodine value of the crude fatty acids of the liver was determined by the method of Wijs following in detail the procedure described by Leathes [1913].

Survival time on the high fat diet.

Rats given the high fat diet ran the typical course of animals deficient in vitamin A, developed xerophthalmia and at autopsy showed the typical infected lesions. The addition of vitamin A to this diet in the form of cod-liver oil (10 mg. daily) or dried cabbage (0·5 g. daily) allowed of normal growth and development in a control group of rats. A group of 15 young rats was given the high fat diet and a control group of 15 was given the standard basal diet. Rats dying before the onset of deficiency symptoms were discarded in analysing the results which are summarised in Table I.

Table I. *Effect of a high fat diet, deficient in vitamin A, on the growth and survival time of the rat.*

Diet	Fat content of diet %	No. of rats in group	Average survival time days	Average maximum increase in wt. g.
Basal	8	12	60	69
High fat	70	11	72	33

[1] It was presumed that after prolonged irradiation little vitamin D would remain in the margarine of the high fat diet.

The growth rate of the rats on the high fat diet was roughly only half that of the control group. Since it is possible that a slower rate of growth resulted in a lessened demand for vitamin A, it becomes impossible to draw any absolute conclusion from the experiment. The high fat group survived for a slightly longer period, on the average, than the control group, which may quite possibly have been due to the slower rate of growth or less possibly to the fact that the high fat diet, though obviously very deficient in vitamin A, contained slightly more than the control basal diet. The experiment on these lines was not however continued further, for the preliminary result appeared to show quite clearly that an excessive amount of fat in the diet had not made excessive demands on the vitamin A reserves such as might be expected if vitamin A were directly related to the metabolism of the fatty acids. If such were not the case the survival time of the high fat group would presumably have been much less than that of the basal group, whereas it was slightly longer.

Loss of body fat in vitamin A deficiency.

Three groups of young rats were given the basal, low fat diet. One group (Table II (1)) received vitamin A in the form of cod-liver oil (10 mg. daily) and the amount of diet eaten was not limited; a second group (2) received a similar diet, but the amount ingested was restricted to the average amount consumed by a third group ((4), (5) and (6)) which received no vitamin A. Rats receiving vitamin A were killed at times corresponding with the spontaneous deaths of rats receiving the basal diet alone. A number of rats ((4) and (5)) in the third group (not receiving vitamin A) were killed during the earlier stages of deficiency symptoms. The remainder of this group were continued on the diet until death occurred. Two other groups of rats ((3) and (7)) were given the high fat diet, with or without the addition of vitamin A. The rats in the group receiving vitamin A were killed at times corresponding with the spontaneous deaths of the vitamin A-deficient group.

Estimations of the total body fat were made in all cases. The results are summarised in Table II.

Table II. *The effect of vitamin A deficiency compared with that of restriction of total food intake on the fat content of the rat.*

Diet	No. of rats in group	Average wt. at death g.	Weight curve	Total body fat %	Remarks
+vitamin A.					
(1) Basal	9	108	Rising	10·8	Killed when rats of group (6) died
(2) Basal	9	68	Falling rapidly	3·5	Food intake restricted to that of group (6). Killed when rats of group (6) died
(3) High fat	6	125	Rising	17·2	Killed when rats of group (7) died
No vitamin A.					
(4) Basal	8	84	Level	9·0	Killed at early stage of vitamin A deficiency
(5) Basal	5	79	Falling slowly	7·0	Killed at later stage of deficiency—7 days later (on average) than group (4)
(6) Basal	9	64	Falling rapidly	2·6	Spontaneous death. Terminal stage of deficiency
(7) High fat	6	54	Falling rapidly	2·8	Spontaneous death. Terminal stage of deficiency

It will be noted that in the early stage of vitamin A deficiency (Table II (4)) there was a slight fall in the amount of body fat, and that this continued to fall gradually with the gradual loss in weight (5). With the rapid loss of weight which closely preceded death the loss of body fat was very rapid. It is to be noted however that this loss was not significantly greater than where the loss of body fat was due to a simple restriction in the amount of food eaten (*cf.* (2) and (6)).

As would be expected the rats receiving the high fat diet had more body fat than those of the group receiving the standard basal diet when, in each case, vitamin A was given (*cf.* (1) and (3)), but it is interesting to note that in the absence of vitamin A the total body fat reaches a similar low level in each group at the time of death (*cf.* (6) and (7)). There is not the slightest indication here of a failure of the animal, deficient in vitamin A, to utilise its available fat reserves to the fullest extent. Loss of weight whether produced by underfeeding or by vitamin A deficiency has resulted in a mobilisation and presumably the oxidation of the greater part of the fat originally present in the body before the decline in weight set in.

The iodine value of the liver fats in vitamin A deficiency.

It seemed desirable to find whether the first stage of fat metabolism, that of desaturation in the liver, differed in the course of vitamin A deficiency from that observed in the underfed animal, where the iodine value of the liver fat rises as the amount of fat in the liver falls. To this end groups of rats similar to those of the preceding section were used with an additional group, receiving a complete diet, in which the amount of liver fat was reduced by artificial infection with a pathogenic strain of haemolytic streptococci or pathogenic organisms of

Table III. *The effect of vitamin A deficiency, underfeeding and artificial infection on the iodine value of the liver fatty acids.*

Diet	Degree and type of infection	No. of rats in group	Liver (average values) Weight g.	Fat content %	Iodine value of fat	Remarks
+ vitamin A.						
(1) Basal	None	17	6·3	4·1	131	Killed when rats of group (7) died
(2) Basal	None	8	3·8	2·7	161	Food intake restricted to that of group (7). Killed when rats of group (7) died
(3) Basal	Severe. Inoculated	11	4·0	2·7	169	Spontaneous death
(4) High fat	None	6	6·4	7·7	130	Killed when rats of group (8) died
No vitamin A.						
(5) Basal	None macroscopically	22	4·3	4·4	124	Killed at early stage of deficiency. Weight stationary
(6) Basal	Mild. Autogenous	10	3·0	3·9	127	Spontaneous death but only moderate loss of weight
(7) Basal	Severe. Autogenous	16	2·6	2·4	160	Spontaneous death. Rapid loss of weight
(8) High fat	Severe. Autogenous	6	2·2	3·0	169	Spontaneous death. Rapid loss of weight

the colon group. The iodine value of the liver fat was estimated in each rat. The results are summarised in Table III.

It will be noted (Table III (5)) that in the very early stages of vitamin A deficiency there was no decrease in the percentage amount of liver fat; in fact it was slightly higher than that of the control group; the iodine value of the liver fat was, as would be expected, somewhat lower than that of the control group (*cf.* (1) and (5)). Group (6) consists of rats which died as a result of vitamin A deficiency, but death was preceded by only a moderate loss in weight, and the amount of liver fat was only slightly below the normal level. This group was particularly interesting, for the iodine value of the liver fat was practically the same as that of the controls (*cf.* (1) and (6)). On the other hand, when the amount of liver fat was much reduced as a result of vitamin A deficiency (group (7)), restriction of food intake (group (2)) or infection (group (3)), the iodine value of the fat rose to roughly the same level in each case.

Group (4), which received the high fat diet and vitamin A, showed a much higher liver fat content than group (1), which received the standard basal diet and vitamin A. It is particularly to be noted that group (8), given the high fat diet without vitamin A, showed a decrease in the amount of liver fat to a level not much above that of group (7), though the amount of fat in the liver before the stage of vitamin A deficiency was reached was, in the two groups, very different.

It is clear from these results that the iodine value of the liver fat has risen as the amount of fat in the liver has fallen, a fact which Hartley and Mavrogordato [1908] first observed, and which was confirmed in human livers by Imrie [1914]. The later stages of vitamin A deficiency, underfeeding and severe infection have all produced a diminution in the amount of fat in the liver,which in effect involves the saturated more than the unsaturated fatty acids and thus results in a rise in the iodine value of the residual fatty acids. The effect of vitamin A deficiency, was, in this connection, no different from that when a diminution in the amount of liver fat was produced by other means. It would seem that the liver, devoid of vitamin A, is able to desaturate the fats presented to it, or otherwise it would be expected that fatty acids of a lower iodine value would be found in it than is the case where a loss of fat occurs in the liver containing vitamin A. Removal of fat appears to proceed quite efficiently even when the diet is very rich in fat, a fact which strongly suggests that vitamin A is not concerned with this process.

Discussion.

Though these findings throw no new light on the physiology of vitamin A, they assist, in conjunction with the findings of other workers, in excluding some of the more obvious features of fat metabolism from a probable association with vitamin A. Large quantities of fat can be absorbed in the absence of vitamin A from the diet, and mobilisation, desaturation in the liver and oxidation apparently proceed normally when the vitamin A reserves of the animal are exhausted.

If it is conjectured that vitamin A is concerned with some aspect of fat metabolism it is probably not with that of the simple fatty acids but of the more complex lipins. Monaghan and Schmitt [1932] have suggested that vitamin A is concerned with phospholipin metabolism on the grounds of experiments *in vitro* in which it was shown that both the unsaponifiable fraction of cod-liver oil and carotene inhibit the oxidation of linoleic acid. Linoleic and other more highly unsaturated fatty acids are found chiefly in the phospholipins of the body, and the preliminary results of these workers suggest a large diminution in the phospholipin content of rats deficient in vitamin A. There is however no

indication in the present results (see Table III) that, as regards the liver, there is any significant difference in the content of unsaturated fatty acids between the vitamin A-deficient rat and the rat in which loss of fat has occurred from other causes in the presence of vitamin A.

There is another discovery which originally suggested a possible connection between vitamin A and lipin metabolism. Mellanby [1926] found that in the vitamin A-deficient dog there was a widespread degeneration of nerve fibres which was readily shown by staining with agents fixed by free fat. He has suggested that this breakdown of the medullary sheath may be the primary lesion of vitamin A deficiency, the keratinisation of epithelium and infection being a secondary trophic change. The intimate structural relationship of the lipins to the nerve myelin sheath suggested to me that some alteration in their metabolism might be the factor leading to degeneration. It was later shown by Mellanby [1933], however, that this degeneration only affected the afferent nerves, whilst the efferent nerves were left relatively intact, and it does not appear probable that if the lesion were due to a chemical change in the medullary sheath it would affect one physiological type of nerve only. It is perhaps not without significance however that the presence of carotene in the nerve fibre has been demonstrated [Monaghan and Schmitt, 1930–31]. These suggestions are obviously speculative, but they indicate the possibility that though vitamin A is probably not related to any of the simpler manifestations, it may yet prove to be related to some more obscure process of fat metabolism.

SUMMARY.

1. No evidence was obtained that a diet very rich in fat accelerates the utilisation of vitamin A in the body.

2. The diminution in the total crude fat of the whole body is not significantly different in the vitamin A-deficient rat from that in the rat on a complete diet whose food intake is restricted to that of the deficient animal.

3. A rise in the iodine value of the fatty acids of the liver, coincident with a fall in the percentage amount of fat in the liver, occurs in the end stages of vitamin A deficiency. The rise is of a similar order to that observed where a similar loss of liver fat has been produced by a restriction of food intake or by infection.

My best thanks are due to Prof. E. Mellanby and to Prof. J. B. Leathes for their interest in this work and to Dr L. Colebrook for supplying a strain of streptococcus pathogenic to rats. The author is in receipt of a personal grant from the Medical Research Council.

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VI. RESEARCHES ON FAT METABOLISM. II.

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(*Received November 18th, 1933.*)

(1) IT was shown in the first paper of this series [Verkade *et al.*, 1933] that with two healthy subjects (V. and v. d. L.) considerable quantities of undecanedioic acid were excreted in the urine after administration of triundecylin. In the human organism the terminal methyl group of the undecoic acid, the component acid of triundecylin, is thus oxidised—and as we shall see later, very readily—to a carboxyl group. The term *ω-oxidation* was introduced by us for this type of oxidation

$$CH_3.(CH_2)_9.COOH \rightarrow HOOC.(CH_2)_9.COOH.$$

The present communication deals with the question whether the administration of chemically pure simple triglycerides of other, higher and lower, odd and even saturated fatty acids leads similarly to the excretion of the corresponding dicarboxylic acids and if so, to what extent.

Influence of simultaneous administration of carbohydrate.

(2) During our original experiments with triundecylin this formed part of a diet which was very poor in carbohydrate; the subject V., *e.g.*, then received 23 g. carbohydrate, 136 g. protein, 192 g. ordinary food fat and 100 g. triundecylin. In a continued study of the *diaciduria* (thus we term the phenomenon of excretion of dicarboxylic or dioic acids in the urine) produced by triundecylin we have discovered that addition of a liberal amount of carbohydrate to the diet leads with most people, among them also the subjects V. and v. d. L., to a pronounced rise of the dioic acid excretion. We have repeatedly taken advantage of this noteworthy and definitely important fact, which very probably points to a function of the liver in the processes here in question and which will be discussed fully in a later communication, in the investigations which will be dealt with in this paper.

Because of their general plan the simple experiments described in this paper can only serve for a preliminary orientation; we shall return to this later. For this reason also we have considered it superfluous to indicate in detail the composition of the diet administered and the distribution of it over the day for all the experiments. We have frequently regarded it as sufficient to mention only a few brief data on the point.

EXPERIMENTAL.

Tricaprylin.

(3) The tricaprylin used was prepared in the way described elsewhere [Verkade *et al.*, 1932] and refined in the way commonly employed technically. Caprylic (octoic) acid with a setting-point of 16·1°, which was isolated from coconut fat *via* the ethyl ester, served as our starting-material. The setting-point of an earlier caprylic acid specimen purified by us with the greatest

possible care, which had at that time to serve for precision measurements, lay only a little higher, namely at 16·35°; Garner and Randall [1924] give the same setting-point for a similar product. The saponification number of the fat obtained was 357·3 (calculated 357·8) and the setting-point 9·45°; we find the values 8–8·3° [Scheij, 1899], 9° [Jaeger, 1917] and 8·3° [Loskit, 1928] for the melting-point of tricaprylin in the literature.

A diet which contained 100 g. tricaprylin as well as amongst other things a liberal amount of carbohydrate, was administered to the healthy subject V. The fat was taken mixed with coffee in portions of 20 g. at practically equal intervals in the course of 9 hours; the carbohydrate was also distributed over this period in several portions. The urine of the subject was carefully collected from the time of partaking of the first portion of fat and indeed up to and including that passed immediately after rising on the next day; on the basis of researches to be published later we knew that collection of the urine for a longer time was unnecessary.

The collected urine was made strongly alkaline with KOH and then evaporated as far as possible, and the residue was heated for a considerable time on the water-bath. Hydrolysis of the hippuric acid present takes place as a result. The removal of this acid, which is relatively little soluble in cold water, facilitates the isolation in a pure state of the dicarboxylic acid sought, especially if—as, for example, in the isolation of sebacic acid discussed on p. 34—extraction with benzene is not employed; hippuric acid is only very slightly soluble in boiling benzene. It is almost certain that compounds of dicarboxylic acids with glycine, glucuronic acid *etc.* if present would also be hydrolysed. The residue after evaporation was taken up in water, acidified with hydrochloric acid and continuously extracted with ether for a considerable time. The ethereal extract was filtered and the residue obtained after distilling off the ether was dried *in vacuo*. It was subsequently extracted several times with 50 ml. of boiling benzene. The mass of crystals gradually separating from the combined cold benzene solutions contains practically all the suberic acid present; this acid is indeed practically insoluble in cold benzene [Verkade and Coops, 1930, 2]. The crystals were filtered off and taken up in hot water. The hot aqueous solution was treated with a small amount of adsorption charcoal and then filtered through a small glass filter. From this solution evaporated to about 15 ml. 0·18 g. of practically pure suberic acid (octanedioic acid) separated out on standing in the ice-chest. The M.P. of this product was 139–141°; the mixed M.P. with a specimen of suberic acid prepared in the usual way from castor oil [Verkade, 1927] and melting at 140–141° was 139–141°.

0·1009 g. required 11·79 ml. 0·0974 *N* NaOH; equiv. wt. 87·9; calc. 87·06.

Tricaprylin was the last triglyceride we examined. As appears from the table on p. 37 we had good reason to expect that only very small quantities of suberic acid would be excreted after administration of this substance [*cf.* Verkade and van der Lee, 1933]. We therefore gave the subject v. d. L.—the latter generally shows the greatest tendency to diaciduria of all subjects who have been at our disposal—100 g. of tricaprylin in a single portion together with 160 g. of bread, 25 g. of sugar and 10 g. of butter shortly after rising. Our intention in doing this was naturally to flood the body with resorbed caprylic acid—we shall not consider the question of the form in which this circulates in the blood—and thus to provoke accumulation of suberic acid in the blood and excretion thereof in the urine. The experiment with the subject V. described above has taught us later that this forced administration of tricaprylin was unnecessary. Diarrhoea occurred in the evening as was indeed not surprising;

investigation of the faeces showed, however, that by far the greater part of the fat administered had been resorbed. The urine was again collected up to the next morning while the subject was still fasting.

In this experiment 0·12 g. of practically white suberic acid, M.P. 139–140°, was isolated from the urine; the mixed M.P. was also 139–140°.

(Found: equiv. wt. 86·8, C 55·4, 55·5; H 7·7, 7·7%: calc. for $C_8H_{14}O_4$: equiv. wt. 87·06; C 55·14; H 8·11 %.)

Trinonylin.

(4) The preparation and purification of the trinonylin employed has already been described elsewhere [Verkade *et al.*, 1932]. The setting-point of this fat was 8·7°, the saponification number 328·7 (calc. 328·5).

To the subject v. d. L. a diet was administered which contained besides 100 g. of trinonylin a liberal amount of carbohydrate in the form of bread and sugar. The fat was taken as such in portions of 20 g. at nearly equal intervals in the course of 10½ hours; the carbohydrate was also taken in portions a short time before the fat. The urine was again collected up to the following morning.

The ethereal extract of the urine obtained in the way described on p. 32 and dried *in vacuo* was extracted several times with 50 ml. of boiling benzene. The odd acids of the oxalic acid series, with the exception of the two lowest members, are readily soluble in boiling benzene and are thus rapidly extracted in this way even when present in somewhat larger quantities; on the other hand they are very sparingly soluble in cold benzene; azelaic acid, *e.g.* only to the extent of 9 mg. in 100 g. of benzene at 25° [Verkade and Coops, 1930, 2]. The mass of crystals separated from the combined ice-cold benzene solutions was filtered off at the pump and taken up in hot water. The hot aqueous solution was treated with some adsorption charcoal and concentrated to about 75 ml. On cooling 0·60 g. pure azelaic acid (nonanedioic acid) separated out. The M.P. of this product was 104–105°; the mixed M.P. with a specimen of azelaic acid prepared in the usual way from castor oil [Verkade, 1927] and melting at 106·5–107·5° was 105–106°. A further 0·05 g. of the same acid was obtained from the benzene and aqueous mother-liquors. Thus a total of 0·65 g. of azelaic acid could be isolated from the urine.

(0·2020 g. required 20·53 ml. 0·1041 *N* NaOH: equiv. wt. 94·5; calc. 94·06.)

A similar experiment was made with the subject V. The latter excreted 0·35 g. of azelaic acid in the urine after taking 85 g. of trinonylin.

(Found: C, 57·4, 57·6; H, 8·3, 8·4 %; calc. for $C_9H_{16}O_4$: C, 57·41; H, 8·56 %.)

Tricaprin.

(5) The tricaprin used was prepared in the manner described elsewhere and purified by recrystallisation from a mixture of alcohol and ether. Capric (decoic) acid setting at 31·35°, which was obtained from pure *n*-octylmalonic acid, served as starting material. The fat obtained set at 30·95°, thus below body-temperature, and was consequently applicable as such to the experiments in hand (see on the other hand sections (7) and (8)); the values 31·1° [Scheij, 1899; Jaeger, 1917] and 31·0° [Loskit, 1928] are given for the M.P. of tricaprin in the literature. The saponification number of the fat was 302·7 (calc. 303·4).

The experiments were here carried out with the standard technique which we apply to other work and which will be discussed rather more fully in a later paper.

Shortly after rising 25 g. of tricaprin melted up in coffee together with a liberal amount of carbohydrate were administered to the sober, healthy subject V. The urine was collected at intervals of 1–2 hours and the presence of sebacic acid was tested for in each portion by treating the urine with a concentrated mineral acid, *e.g.* phosphoric acid; even with very small concentrations of sebacic acid the latter then separates out as micro-crystals in a characteristic way. The collection of the urine was continued with the subject still fasting until two successive portions gave a negative reaction.

The ethereal extract obtained in the manner described on p. 32 was extracted with boiling water, and the filtered aqueous solution was evaporated to about 50 ml.; on cooling practically pure sebacic acid (decanedioic acid) crystallised out. This acid weighed 0·55 g. and melted at 129·5–130·5°; the mixed M.P. with a specimen of sebacic acid prepared in the usual way from castor oil and melting at 132·5–133·5° was 132–133°.

(0·1202 g. required 11·26 ml. 0·1046 *N* NaOH: equiv. wt. 102·0; calc. 101·08.)

In another similar experiment this subject excreted 0·11 g. of sebacic acid in the urine.

Two similar experiments were made with the subject v. d. L. With the latter 1·10 g. and 0·29 g. of sebacic acid were respectively excreted in the urine after partaking of 25 g. of tricaprin together with a liberal amount of carbohydrate. Below are given some analytical data for the acid isolated in the last experiment, recrystallised once more from water after treatment with some adsorption charcoal and then melting at 131·5–133°.

(Found: equiv. wt. 101·7; C, 59·6, 55·6; H, 8·8, 8·8 %: calc. for $C_{10}H_{18}O_4$: equiv. wt. 101·08; C, 59·36; H, 8·98 %.)

Triundecylin.

(6) It is here sufficient to state that, employing the above mentioned standard technique, with the administration of 25 g. of triundecylin together with a liberal amount of carbohydrate, amounts of undecanedioic acid, varying from 0·29 g. to 0·66 g. and from 0·60 g. to 1·59 g. were excreted by the subjects V. and v. d. L. respectively. These experiments will be discussed in detail in the communication in which we deal with the rôle of the carbohydrate.

Trilaurin.

(7) The trilaurin required was obtained in a yield of more than 85 % by crystallising tangkallak fat (from *Litsaea sebifera* Bl.) several times from a mixture of 2 vols. 96 % alcohol and 1 vol. ether [van Eldik Thieme, 1911]. Its saponification number was 263·0 (calc. 263·5). The M.P. of trilaurin is 46·2°.

By melting together 1 part of trilaurin with 1 part of coconut fat a mixture was obtained which was completely liquid at body-temperature. The use of such a mixture appeared to us to be necessary to obtain a good adsorption of the fat. On administering trilaurin as such, completely melted up in coffee or thin broth, it was excreted to an appreciable extent in the faeces in the form of large and small solid pieces. For two reasons we have used coconut fat for the purpose mentioned and not a fatty oil setting only at low temperatures. Firstly it is well known in the food fat industry that the addition of coconut fat is particularly suitable for lowering the M.P. of a fat; secondly coconut fat contains amongst its component fatty acids an appreciable amount (about 50 %) of lauric acid, although in the form of mixed glycerides. Against this addition of coconut fat one can *a priori* raise the objection that this fat contains as com-

ponent fatty acids various other higher and lower acids of the same series in varying though always much smaller amounts. One may ask whether administration of the fat mixture in question might lead to the excretion of a mixture of several dicarboxylic acids in the urine, so that the isolation in the pure state of dodecanedioic acid possibly present and the proof of the origin thereof as an ω-oxidation product of lauric acid might be made much more difficult. From what follows it will be seen that this objection is of a purely theoretical nature and, taking into account the quantitative composition of coconut fat [see *e.g.* Taylor and Clarke, 1927], we get an indication why this is so. Reference may be made here to the third paper of this series.

We have administered this fat mixture to various subjects, finally, since no positive results were obtained, in very large quantities. We shall only discuss two of these experiments here, one of them somewhat more fully.

225 g. of the fat mixture in question were administered in the course of 8 hours as part of a diet very poor in carbohydrate to the healthy subject B., to whom on the preceding day no food whatever had been given. We succeeded in isolating from the urine collected up to the following morning a few mg. of a substance which melted at 118–122° and the mixed M.P. of which with a specimen of dodecanedioic acid, prepared by the method of Walker and Lumsden [1901] and melting at 125–127°, was 119–124°. After recrystallising once more from boiling benzene this substance melted at 122·5–125·5°. Taking the solubility relations of this substance also into account—it was very slightly soluble in cold water and cold benzene [see Verkade and Coops, 1930, 2], more readily in cold ether and boiling water and readily in boiling benzene—the conclusion is justified that we were very probably dealing with dodecanedioic acid. The amount of material was quite inadequate for a more definite confirmation by titration or elementary micro-analysis.

In the second experiment 190 g. of the same fat mixture were administered to the same subject in the course of 8½ hours, together with a liberal amount of carbohydrate in the form of bread and sugar. The composition of the diet and the distribution of it over the day are indicated in the following table:

Time	Bread g.	Sugar g.	Fat mixture g.
9.15	40	8	—
9.40	—	—	30
11.00	—	—	30
11.10	40	—	—
12.15	—	—	30
13.15	80	8	—
13.30	—	—	25
14.45	—	—	25
16.45	30	—	25
18.00	—	—	25
18.55	70	—	—
	260	16	190

Each portion was taken after being completely melted up in thin broth; taking the large amount of fat was greatly facilitated by adding some lemon juice and eating a few grapes. On working up the urine, again collected until the following morning, indications of the presence of only a very small amount of dodecanedioic acid were obtained.

Notwithstanding the administration of tea and of *pulvis opii*—in the second experiment, for example, the subject received 50 mg. of the latter each time at 12 hours 30, 17 hours and 23 hours—diarrhoea occurred in both experiments,

which can indeed cause no surprise. It occurred, however, only late at night and it is therefore quite certain that a very considerable part of the fat mixture administered had been adsorbed. That no appreciable excretion of dodecanedioic acid in the urine took place, in spite of the large amount of fat adsorbed in these experiments, is very striking; for example, for comparison with the result of the second experiment it should be mentioned that the subject B. excreted a large amount (namely 1·05 g. and 0·45 g. respectively) of undecanedioic acid on the administration of only 25 g. of triundecylin together with a liberal amount of carbohydrate employing the standard technique in the tests. The same remarkable difference in behaviour between trilaurin and the lower triglycerides was observed with other subjects, among them the subject V.

Since natural products (tangkallak fat and coconut fat) served as the starting materials in the experiments discussed in this section, for the sake of completeness we shall deal with the question whether these products possibly contained preformed dodecanedioic acid in some form or other so that the traces of this acid, which were present in the urine of the subject, might be derived from this. This question is really of no importance to us. Indeed in the case of a confirmatory answer the contrast between trilaurin and the lower triglycerides, to which we have just drawn attention, becomes, if possible, still more pronounced. However, a negative answer is already clearly implied in the fact that—as will be shown in later communications partly from data in the literature [see, among others, Baer and Blum, 1908; Mori, 1918; Flaschenträger, 1927], partly from our own investigations—animal and human organisms are able to burn considerable amounts of the higher normal saturated dicarboxylic acids in a day, combined with the consideration that the trilaurin obtained by repeated crystallisation of tangkallak fat from alcohol-ether and the coconut fat (both products which were repeatedly and carefully investigated) in no case contained appreciable amounts of these acids.

Tritridecylin.

(8) The preparation and purification of the tritridecylin employed has already been described elsewhere. The saponification number of this fat was 246·1 (calc. 247·3) and the setting-point 42·65°.

To each of the subjects V. and v. d. L. 25 g. of this fat completely melted up in thin broth or coffee together with a liberal amount of carbohydrate were administered, employing the standard technique. No tridecanedioic acid could be detected in the urine collected up to the next morning. In this case too, however, a considerable part of the fat supplied was excreted in the faeces in the form of large and small solid pieces.

In order to lower the melting-point below body temperature we next melted tritridecylin together with the same weight of coconut fat. From the experiments discussed in section (7) we knew that, after taking coconut fat, no substances are excreted in the urine which might interfere with the isolation in the pure state of any possibly excreted tridecanedioic acid. The subject V. partook in the usual way of 50 g. of this mixture, again together with a liberal amount of carbohydrate. No tridecanedioic acid was excreted in the urine.

Finally a mixture was made of 25 g. of tritridecylin with 10 g. of paraffin oil; such a mixture shows very little tendency to crystallise at body temperature. In the preceding experiment tritridecylin formed only 50 % of the weight of a fat mixture which could be completely adsorbed in the intestines; in the mixture just mentioned on the other hand tritridecylin is the only absorbable compound,

present in it to an amount of 70 %. It therefore appeared to us conceivable, and even not improbable, that on administering such a mixture tritridecylin would be absorbed at a greater rate than on administration of the mixture of tritridecylin and coconut fat, and consequently that there would be a greater chance of the accumulation of tridecanedioic acid in the blood and of the excretion of this substance in the urine. On the contrary, it is true that a reduction of the absorbability of tritridecylin as a result of the simultaneous presence of the paraffin oil may also be conceived. We believe on good grounds that any appreciable influence of the small amount of paraffin oil in this sense may be excluded; for example, it will be shown in a communication to be published later that the velocity of saponification of the simple triglycerides under the influence of pancreas lipase is not altered by the addition of paraffin oil. The subject V. partook of the mixture in question, completely melted up in thin broth together with a liberal amount of carbohydrate; no hindrance whatever due to the presence of the paraffin oil was experienced. Again no tridecanedioic acid appeared to be present in the urine collected up to the next morning.

Discussion.

(9) As appears from the preceding sections, administration under, as far as possible, corresponding conditions of different simple saturated triglycerides to one and the same subject leads to excretion of dioic (dicarboxylic) acid in the urine in very different amounts.

We only found a very considerable excretion of dioic acid after tricaprin and triundecylin had been taken. The amount of dioic acid excreted on the administration of always the same amount of the same triglyceride to one and the same subject under externally similar conditions can vary fairly considerably, while different subjects can also behave very differently with respect to the same triglyceride. We shall go into these facts, concerning which a few data are already to be found above, in later communications. It is consequently impossible to indicate yet on the basis of the results of the few experiments discussed above whether triundecylin or tricaprin has the greater tendency to cause dioic acid-acidosis and diaciduria; in other words—as we shall term it—which of the two is the more *diacidogenic*. Even after further experience obtained by us in the continuation of the work here described, we do not yet wish to state an opinion on this point. Trinonylin is certainly considerably less diacidogenic than the triglycerides just discussed, and in tricaprylin the diacidogenic properties are almost certainly still less developed. With the two higher triglycerides investigated by us, tritridecylin and trilaurin, dioic acid was either not found in the urine or at most only in traces, in spite of the selection of subjects with a considerable tendency to diaciduria.

A comparative review of the diacidogenic properties of the triglycerides investigated, based on the experiments described in this paper and therefore obviously being only of a preliminary nature, is given here.

	Term-number*	
Tritridecylin	12	Not diacidogenic
Trilaurin	11	Practically not diacidogenic
Triundecylin	10	Strongly diacidogenic
Tricaprin	9	Strongly diacidogenic
Trinonylin	8	Rather weakly diacidogenic
Tricaprylin	7	Weakly diacidogenic

* See Verkade and Coops [1930, 1].

The results of our experiments with tricaprylin justify the question whether the diacidogenic properties of the lower triglycerides only decrease slowly on descending the series and whether it will therefore, for example, be possible to prove the excretion of pimelic acid (heptanedioic acid) in the urine after the administration of triheptylin to suitable subjects. It did not at the time appear to us necessary to study this point.

On the other hand we can indeed say with certainty that with the triglycerides of higher term-number [Verkade and Coops, 1930, 1] than those we investigated (>12)—we leave out of consideration the difficulties which these substances would lead to on account of their high M.P.—there will be no question of dioic acid-acidosis or dioic acid excretion in the urine.

However puzzling this remarkable course of the diacidogenic properties may still be in the main, one thing appears very clearly, namely, that there is definitely no question of a contrast in this respect between the triglycerides derived from acids with odd and even numbers of C atoms; the behaviour of the triglycerides with the term-numbers 9 and 10, 11 and 12 may be compared.

In succeeding communications we shall show that the normal saturated fatty acids—we shall at present deal only with these—are catabolised in a hitherto absolutely unknown way besides the classical way of the β-oxidation discovered by Knoop, which from now onwards we shall call *one-sided β-oxidation*. By ω-oxidation of the fatty acid the corresponding dioic(dicarboxylic) acid is formed which is then further catabolised *via* lower dioic acids formed by *two-sided β-oxidation*. It may be remarked in passing that we in no way wish to exclude the possibility of the occurrence of still further mechanisms for the degradation of these fatty acids.

It is a fact that practically no one—and in our opinion certainly rightly—doubts the degradation of saturated fatty acids by one-sided β-oxidation. The arguments in favour of this way of degradation—one can hardly speak of proofs—are, however, really of little significance. The only indication in this direction with the fatty acids with even numbers of carbon atoms, the acid components of our normal food fats, really consists in the formation from them of ketonic bodies under various conditions, which need not be enumerated here, and with the fatty acids with odd numbers of carbon atoms in the non-occurrence of ketonic bodies under these same conditions. Finally the greatest support for the correctness of the view in question is derived from experience obtained with derivatives of the fatty acids, *e.g.* with the ω-phenyl-substituted fatty acids by Knoop, with the fatty acids containing a methyl(benzenesulphonyl)amino-group by Flaschenträger, *etc.* Closer investigation of this way of degradation of the fatty acids proper is quite definitely desirable; in fact such work is included in our programme.

On the other hand in the case of the way of degradation of the fatty acids now discovered by us we have been able to follow the degradation as it were from phase to phase both with the fatty acids with even and those with odd numbers of carbon atoms with the help of simple *in vivo* experiments. As far as the first phase is concerned, the ω-oxidation, the evidence for this statement is to be found in the experimental part of this paper.

(10) We shall naturally have to deal fully in later papers with the question of the factors to which is due the fact that administration of the various simple saturated triglycerides under as far as possible similar conditions to the same healthy subjects leads to accumulation of dioic acid in the blood and excretion of it in the urine in such typically divergent amounts. At present very little can be said with certainty on this subject. The catabolism of fat administered *per os*

takes place through a highly complicated series of processes, which we cannot as yet properly grasp at all even as regards its main features, and each phase of which will occur generally with a characteristic speed for each triglyceride, or each fatty acid, *etc.* Now it is certainly obvious to suppose that it will depend on the velocity relationships of all these processes whether, and if so to what extent, accumulation of dioic acid in the blood will occur after administration of a certain triglyceride and consequently—perhaps on exceeding a certain threshold concentration—excretion of this acid will take place in the urine. Experiments such as those described in this paper can—we already pointed this out expressly in section (2)—only serve for a first orientation with regard to the newly discovered way of degradation of the fatty acids and of the phenomena connected therewith. A deeper insight can only be obtained by comparative investigations of the behaviour of the triglycerides in question in different phases of the total process of fat metabolism. The first results of work carried out in this direction in our laboratory will soon be published.

(11) It may be stated here that by accepting the idea very briefly indicated above with regard to the cause of the characteristic serial change of the diacidogenic properties in successive triglycerides one may, or rather one must, logically conclude that there also exists some analogous change in the ketogenic properties of successive triglycerides, derived from fatty acids with even numbers of carbon atoms. Systematic work in this region has not yet been carried out. There is certainly also here a possibility of interesting and doubtless important investigations with the aid of perfectly defined synthetic fatty acid derivatives; we are meanwhile convinced that it will definitely be very difficult to reach really unambiguous results in this domain.

SUMMARY.

1. Administration under as far as possible corresponding conditions of a series of simple saturated triglycerides to one and the same subject leads to excretion of dicarboxylic acid in the urine in very different amounts.

2. With respect to this ω-oxidation of the component fatty acid there is no contrast between triglycerides derived from acids with odd and even numbers of C atoms.

3. The excretion of dicarboxylic acid is largest with triundecylin and tricaprin; it diminishes on descending the series. No dicarboxylic acid is excreted after administration of the higher triglycerides.

4. The supposed cause of this peculiar serial change of the so-called diacidogenic properties of these successive triglycerides is indicated.

5. It is pointed out that consequently some serial change may or rather must exist in the ketogenic properties of successive triglycerides, derived from fatty acids with even numbers of carbon atoms.

We gladly acknowledge the financial assistance received from the Hoogewerff-Fonds of The Hague and the Erasmus-Stichting of Rotterdam for the purpose of these investigations.

We thank the Afdeeling Handelsmuseum of the Koloniaal Instituut at Amsterdam for the readiness with which tangkallak fat was supplied to us.

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VII. A NEW COLOUR TEST FOR THE DETERMINATION OF VITAMIN A.

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(*Received November 18th, 1933.*)

Since the paper of Carr and Price [1926] much attention has been directed towards the antimony trichloride reaction of vitamin A. Opinions as to the value of this test are not uniform; it is, however, generally emphasised that this colour reaction is not dependable enough for exact determinations; at best it can serve as an orientation within certain limits. In the determination of the vitamin A content of cod-liver oil this colour test yields uncertain readings. The colour of the reaction is not stable; at first the solution is blue but it soon becomes violet and finally red. For measuring the colour intensity of the Carr-Price reaction the Lovibond tintometer is generally adopted. Owing to the rapid colour change it is expedient to accomplish readings within a very short time—10 to 30 seconds. Moreover, the test is not specific for vitamin A, as other carotenoid substances show the same behaviour.

Owing to the drawbacks mentioned, several new tests have been proposed, none of which, however, is satisfactory.

We attempted therefore to devise a reaction characteristic of vitamin A and allowing its exact determination in cod-liver oil. We succeeded in finding an adequate test by a simple modification of the Carr-Price reaction: if the oil containing vitamin A is heated together with catechol and antimony trichloride, both dissolved in chloroform, the blue colour first produced soon turns into a violet-red. The following reagents are needed for the test:

1. Alcohol-free, absolute chloroform (lower alcohols and water impede the reaction).
2. 0·5 % solution of catechol in chloroform.
3. A cold saturated solution of antimony trichloride in chloroform.

The specimen of oil to be tested is first diluted with chloroform. To 1–2 ml. of this solution 1 ml. of the catechol solution and 2–3 ml. of the antimony trichloride solution are added. The test-tube containing the mixture is immediately transferred to a water-bath at 60° for 1–2 minutes. The blue colour first produced changes during this heating into an intense violet-red hue. In the cold this colour is not produced, as the change then takes the same course as without catechol. Subsequent heating of the solution which has undergone the change in the cold does not lead to the production of the violet-red colour; therefore heating must take place immediately after the reagents have been mixed.

Spectroscopic examination of the red fluid shows characteristic absorption bands. Four bands have been observed, two of which are pronounced enough to be measured with the diffraction spectroscope.

1. A broad band at the boundary of green and yellow, having indistinct limits; peak intensity at 552 $m\mu$.

2. A narrower, distinct band between green and blue; peak intensity at 476 $m\mu$.

Besides those mentioned, two more dim bands were observed, one in the blue and the other in the violet.

Solutions of vitamin A irradiated and thus inactivated with ultraviolet light do not give the reaction described; the solution becomes pink at once, and changes into brownish red with a bluish tinge.

Irradiation by means of X-rays has a similar effect: the solution first becomes red, then reddish brown.

As mentioned, the reaction of Carr and Price is given not solely by vitamin A, but also by other carotenoid bodies. By the courtesy of Professor Zechmeister, to whom our thanks are due, we have been able to examine carotene, lycopin, zeaxanthin, capsanthin and physalin. None of these carotenoids gave our reaction; they gave the original blue colour of the Carr-Price test and no change of colour could be observed on heating. We were unable to purchase other carotenoids, which will be studied later.

Thus our reaction makes it possible to differentiate vitamin A from carotene. Moreover, vitamin A can be demonstrated simultaneously with carotene; the red of vitamin A and the blue of carotene blend into a vivid violet.

The same reaction carried out with ergosterol shows the colour change in an inverse succession, the solution first becoming coloured pink which changes slowly into blue. No catechol is needed for this reaction; the colour change is, however, greatly hastened by its presence. Heating does not enhance it, moreover this is disadvantageous; when heated, the solution becomes green, but on cooling the blue colour reappears.

With irradiated ergosterol (vitamin D, vigantol oil) no colour reaction is observed.

The blue colour produced with ergosterol shows an absorption band between yellow and green, having a peak intensity at 595 $m\mu$; another band beginning at about 610 $m\mu$ and showing an increasing intensity towards the red can also be observed.

The catechol reaction of vitamin A is also suitable for quantitative determinations. Under certain conditions the intensity of the violet-red colour is proportional to the concentration of vitamin A and can be measured colorimetrically. A 0·01 % solution of potassium permanganate is used for comparison. A specimen of oil containing vitamin A is dissolved in chloroform, yielding a solution of a known concentration. 1 ml. of this solution is employed, or a smaller quantity is diluted with chloroform exactly to 1 ml. Now 1 ml. of the catechol solution and 3 ml. of the antimony trichloride solution are added and the test-tube is put into the water-bath at 60°. After the solution has become violet-red, it is compared in the colorimeter with the standard solution of potassium permanganate.

The correct performance of this reaction depends on the following conditions.

1. The duration of heating. Too short heating produces a red hue with a bluish tinge, whereas prolonged heating yields a brick-red solution.

2. The concentration of the catechol solution. The colour is different if the solution contains more or less than 0·5 % of catechol. More dilute solutions tend to produce a bluish red tinge, higher concentrations a brown one. Catechol solutions should always be freshly prepared, as solutions older than 24 hours are liable to give bluish tinges.

Of cod-liver oil from 0·1 to 0·5 ml. should be taken. When heated the violet-red colour appears in as short a time as 10 to 15 seconds. The reading

should be accomplished as quickly as possible, for within a short time the colour fades into brown. This change is probably caused by resinification of the oil; not more than 0·5 ml. of oil should therefore be used for the test.

Of vitamin A concentrates we examined two brands, called "Essogen" and "Vogan," respectively. Chloroform dilutions were prepared, which had to be heated for 1 to 2 minutes. As these solutions were examined in high dilutions, their oil contents were very low and consequently their colours lasted much longer, averaging 5 to 10 minutes and even more.

The following are the results of several determinations:

1. Cod-liver oil. (The comparator cell of the Duboscq colorimeter stood at the mark 10.)

0·1 ml. of oil	reading	18·0
0·2 ,,	,,	8·9
0·3 ,,	,,	6·2
0·4 ,,	,,	4·6
0·5 ,,	,,	3·6

2. 0·5 ml. of "Vogan" diluted to 10 ml.

0·1 ml. of solution	reading	10·0
0·2 ,,	,,	5·0
0·3 ,,	,,	3·2
0·4 ,,	,,	2·4
0·5 ,,	,,	2·0

3. 0·1 ml. of "Essogen" diluted to 10 ml.

0·1 ml. of solution	reading	30·0
0·2 ,,	,,	15·2
0·3 ,,	,,	10·0
0·4 ,,	,,	7·3
0·5 ,,	,,	6·0

The values above indicate that with an increase of the quantity of vitamin A the readings proportionally decrease, thus the colour intensity is a linear function of the concentration. Comparison of these values showed that "Vogan" contained 36 times, "Essogen" 60 times as much vitamin A as the cod-liver oil examined.

In another experiment we measured the absorption of our solutions photoelectrically. We employed a cell of 2·9 mm. in diameter. The maximum intensity of the photoelectric current amounted to $83{\cdot}5.10^{-7}$ amperes. 0·1 to 1·0 ml. of a 1 : 20 solution of Vogan was used. The results are shown in the following table:

ml. of solution	Current intensity 10^{-7} amp.	Absorption $83{\cdot}5/x$
0·1	47·5	1·8
0·2	39·5	2·1
0·3	28·5	2·9
0·4	22·5	3·7
0·5	18·0	4·6
0·6	13·5	6·2
0·7	10·5	7·9
0·8	7·0	11·8
0·9	5·5	15·2
1·0	2·5	33·4

Spectrophotometric examination of the colouring matter is being carried out. The relation of these data to the biological potency of the preparations examined is reserved for further study.

SUMMARY.

The reaction with catechol as described is an advance in two respects.

1. It makes possible differentiation of vitamin A from carotenoid substances.

2. The violet-red colour, similar to that of a dilute solution of potassium permanganate, is more stable than the blue colour obtained according to the Carr and Price method.

REFERENCE.

Carr and Price (1926). *Biochem. J.* **20**, 297.

VIII. POLARIMETRIC DETERMINATION OF GLUCONIC ACID.

By THOMAS ARCHIBALD BENNET-CLARK.

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(*Received November 22nd, 1933.*)

The specific rotation of malic, tartaric and other α-hydroxy-acids is markedly increased by the formation of complex compounds with uranium salts and with molybdates, tungstates, *etc.* Use is made of this phenomenon in the estimation of optically active malates and tartrates in plant material [Auerbach and Kruger, 1923]. The writer is not aware that this phenomenon has been made use of in the estimation of gluconic acid. It can, however, be conveniently applied to this purpose.

Experimental.

Calcium gluconate recrystallised from water was dried at 100° and a 0·1 *N* (2·15 %) solution was prepared. 5 ml. samples of this stock solution were mixed with 2·0, 5·0, 10·0 and 15·0 ml. respectively of saturated ammonium molybdate solution; 2·0 ml. of glacial acetic acid were added, and each sample was made up to 25 ml. with distilled water. The rotations of these solutions were measured at various time intervals after mixing the reagents.

The results of nine typical experiments are given in Table I. It is found that the rotation of the gluconate-molybdate mixture is at its maximum value as soon after mixing of the reagents as it is possible to take readings (about 5 minutes). The rotation decreases during the first two hours to a value which is maintained almost unchanged for some days. It will be noted from Table I that the rotation of the gluconate-molybdate mixture attains its maximum value when 5 ml. of saturated ammonium molybdate are present in 25 ml. of solution. Trebling the concentration of molybdate only brings about a 3 % decrease in rotation, and halving the molybdate concentration correspondingly brings about a reduction in rotation of about 10 %.

Table I.

	ml. saturated molybdate per 25 ml. of solution	$[\alpha]_D^{25°}$ *		
		1 hour	3 hours	24 hours
I	2·0	343°	314°	—
II	2·0	331	—	—
III	5·0	372	361	361°
IV	5·0	373	—	366
V	5·0	371	—	—
VI	10·0	384	—	—
VII	10·0	362	352	—
VIII	15·0	362	350	—
IX	15·0	347	—	—

* Calculated with respect to quantity of total gluconic acid.

On the basis of these and other similar results, it was decided to treat gluconic acid or gluconate solutions in the following standard manner. 8 ml. of the gluconate solution are mixed with 5 ml. of saturated ammonium molybdate and 2 ml. of glacial acetic acid, and the mixture is allowed to stand for at least 3 hours in darkness. The rotation is then measured. Under these conditions, as the figures in Table II show, the relationship between gluconate content and rotation is almost linear when the gluconic acid content is less than 1·5 % and deviates only slightly at concentrations up to 3 %. In general, plant material containing gluconates or gluconic acid also contains carbohydrate. The specific rotation of glucose is not directly affected by treatment with the molybdate reagent, but relatively concentrated solutions (5 % for example) are fairly rapidly oxidised by the molybdate mixture, which itself becomes dark blue in colour. The rotation of the solution increases probably owing to the formation of gluconic acid among the products of oxidation of the glucose, and polarisation of the darkly coloured solution becomes impossible.

In the presence of sugars therefore the standard procedure mentioned above is varied. A measured volume of the solution is boiled with sufficient excess of NaOH to neutralise the acid and convert any gluconic lactone into sodium gluconate; calcium chloride is added and the solution treated with alcohol to make the alcohol content 80 %. The precipitated calcium salt is filtered, washed and redissolved in water so as to give 8 ml. of solution, to which 5 ml. of molybdate and 2 ml. of glacial acetic acid are then added. After standing 3 hours in the dark the solution is polarised as before.

Table II gives the observed rotations in a 2 dm. tube, and also the specific rotations when solutions of various gluconic acid contents were used in the standard manner described. The quantity of gluconic acid recorded in the left-hand column of the table is the quantity present in the 8 ml. sample used which is made up to 15 ml. by addition of molybdate and acetic acid. Sodium light was used in all cases and the temperature throughout was 22°.

Table II.

Quantity of gluconic acid, mg. in 8 ml.	Observed rotation in 2 dm. tube	Specific rotation
19·6	0·96°	367°
39·2	1·93	369
78·4	3·83	366
117·6	6·10	389
156·8	8·00	381

Intermediate values have been obtained by interpolation. It has been shown that the presence of citric acid does not affect the value of the specific rotation of gluconic acid in a manner comparable with the effect on malate-molybdate mixtures. An 8 ml. sample containing 78·4 mg. gluconic acid and 192 mg. citric acid in standard molybdate mixture gave a rotation of 3·96° in a 2 dm. tube. The specific rotation is therefore 378°.

Table III.

Acid	Specific rotation in presence of molybdate	Specific rotation in presence of uranyl salts
d-Malic	−720 to −770°	+510 to +525°
l-Malic	+720 to +770	−510 to −525
d-Gluconic	+366 to +390	About +80

The gluconate-containing material so far investigated in this laboratory has not contained malic or other optically active acids. It should, however, be possible to deal polarimetrically with such mixtures relatively easily on account of the different magnitudes (and directions) of the enhancements of rotation produced by molybdates and uranyl salts, of which data are given in Table III.

SUMMARY.

1. The specific rotation of *d*-gluconic acid is enhanced about 40-fold by formation of complex salts with molybdates. The value of the enhanced rotation is to a marked extent unaffected by variation in concentration both of gluconic acid and molybdate.

2. The rotations of gluconic acid complexes with uranyl salts and molybdates are in the same direction, in contrast to the behaviour of malates; hence polarimetric analysis of mixtures should be relatively simple.

REFERENCE.

Auerbach and Kruger (1923). *Z. Unters. Nahr. Genussm.* **46**, 97, 179.

IX. THE DETERMINATION OF BROMINE IN NORMAL BLOOD.

By THEODORE FREDERIC DIXON[1].

From the Central Pathological Laboratory of the London County Mental Hospitals.

(*Received November 25th, 1933.*)

During an investigation into the halogen constituents of the blood of psychotics, who, it is stated by Zondek and Bier [1932], have a greatly lowered blood-bromine, it was found that none of the methods available for blood-bromine determination was satisfactory. In particular that of Pincussen and Roman [1929], on which Zondek bases his conclusions, was shown to be subject to gross errors.

In all the methods the blood is first ashed with excess of alkali whereby the bromine is fixed as alkali bromide. The bromine is then liberated by oxidation with $KMnO_4$ and H_3PO_4 [Behr *et al.*, 1931] or HNO_3 [Pincussen and Roman, 1929] and collected in chloroform or carbon tetrachloride, or it is distilled over into potassium iodide solution. In any case it is difficult to avoid oxidising chloride and also to get rid of all traces of oxidising agents even if aeration is resorted to. In the method of Roman the melt is subjected to an alcoholic extraction which is claimed to remove potassium iodide only. The present author has shown, however, that although potassium bromide is only slightly soluble in alcohol the amount (100γ) present in the potassium carbonate melt from 10 ml. normal blood can be quantitatively extracted with 90–95 % alcohol, the bromide and iodide being thus extracted together. Fleischhacker and Scheiderer [1933] have shown that the oxidation with HNO_3 and H_2O_2 is not quantitative. In the Roman method excess of oxidising agent is removed by shaking the $CHCl_3$ layer with ice-cold NaCl solution. Hahn [1933] has shown that NaCl dissolves out some Br as well as the oxidising agents. In any case iodine titration in a water-chloroform mixture is a difficult procedure and not one capable of great accuracy.

According to van der Meulen [1931] bromides can be quantitatively oxidised to bromates by $2\frac{1}{2}$ times the theoretical amount of hypochlorite in the presence of borate buffer and half saturation with NaCl according to the equation

$$3KClO + KBr \rightleftarrows KBrO_3 + 3KCl.$$

The completion of the reaction from left to right is ensured if the oxidation is carried out at 85° and excess hypochlorite is removed by addition of H_2O_2, which in turn is removed by boiling. Addition of bromates to iodides in acid solution results in the liberation of iodine equivalent to 6 times original amount of Br according to the equation

$$HBrO_3 + 6HI \rightleftarrows HBr + 3H_2O + 3I_2.$$

It was found that the above procedure could be modified to deal with quantities of the order of 50γ such as are likely to be encountered in 10 ml. normal blood.

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It has the advantage that the oxidation and titration are carried out in the same vessel and all the oxidising agent is removed. A small amount of chlorate is unavoidably formed but this is allowed for by using controls which have been subjected to the same procedure from the start. Using this method of oxidising bromide and determining it by the iodine liberated it was found possible to evolve a method for determining bromine in 10 ml. normal blood.

Reagents required.

Water distilled from alkali in an all glass apparatus.

10*N* KOH and 10*N* K_2CO_3 purified so as to contain no bromide by von Fellenberg's [1926] method.

Pure ethyl alcohol distilled in all glass apparatus from KOH.

N KClO in *N*/10 KOH. Made by passing 71 g. chlorine gas, cooled and dried, into 1·8 litres of KOH containing 2·2 g. mols. or 123·2 g. KOH, finally making up to 2 litres with water. The KOH is contained in a flask cooled in ice while the chlorine is passing through to minimise formation of $KClO_3$.

N KI, preferably made fresh, contained in a brown glass bottle.

N HCl.

0·5 % starch; acid-washed soluble starch freshly made up each time required.

N H_2O_2.

N/500 sodium thiosulphate solution.

The proposed method for blood is as follows. 10 ml. of oxalated blood are measured into a 300 ml. flask. 0·8 ml. 10*N* KOH, 0·8 ml. 10*N* K_2CO_3 and 30 ml. water are added, and the whole is heated on the water-bath for 4 hours. The mixture is then transferred to a nickel basin of 10 cm. diameter and evaporated to dryness on the water-bath. The basin is dried at 150–160° for 60 mins. and then heated at 420° in the muffle furnace for 2 hours and finally at 500° for ½ hour. The contents are ground up with water by means of a glass rod, evaporated to dryness on the water-bath, dried at 150–160° for 30 mins., and then ignited for ½ hour at 500°. The grinding with water, drying at 160° and igniting at 500° for ½ hour are repeated. During ignition in the muffle the contents of the basin swell up considerably and there is danger of loss unless the surface of the melt is pricked with a finely pointed glass rod, thus allowing tarry vapours to escape. 20 ml. of water are then added to the basin, which is heated for a few minutes on the water-bath. The contents are ground up with a glass rod and then filtered through a 9 cm. Whatman's No. 5 filter-paper previously well washed, into a 9 cm. porcelain basin. The residue is repeatedly extracted with hot and finally cold water until there are about 50 ml. in the porcelain basin which are then evaporated on the water-bath. The porcelain basin is heated on the water-bath to dryness, carefully dried first at 120° for 15 mins., then at 150–160° for 15 mins., and finally ignited in the muffle for 10 mins. at 500°. The contents are dissolved in 15 ml. water and filtered through a 7 cm. Whatman's No. 42 filter-paper into another porcelain basin, the filter being washed several times with *N*/10 K_2CO_3. The solution is evaporated on the water-bath and the residue dried at 150–160° and ignited in the muffle for 5 mins. at 500°. It is then completely dissolved in 1·8 ml. water and alcohol added 5 ml. at a time with continuous stirring, the mixture being well rubbed with a round-ended glass rod, until 15 ml. alcohol have been added. The mixture is stirred until the K_2CO_3 passes into a pasty condition when the alcoholic layer is decanted through a Jena glass sintered filter No. 3 into a 30 or 40 ml. flask. The residue is extracted with two more successive lots of 5 ml. of alcohol. To the potassium carbonate from which the alcohol has been drained

is then added 0·6 ml. water and the above alcoholic extraction is repeated. The contents of the flask are evaporated on the water-bath to dryness, with addition of a couple of pumice granules to facilitate smooth boiling and, if the flask looks very full, under a stream of nitrogen. The last traces of alcohol are removed by heating at 150–160° for 10 mins. To the residue are added 2 ml. water, 1 ml. KClO and 2·5 ml. saturated NaCl, and the mixture is warmed to about 85° on the water-bath and treated with 1 ml. saturated H_3BO_3. The flask is removed after 20 mins. and cooled and then 1 ml. *N* H_2O_2 added, after which the contents are gently boiled for 5 mins. The flask is cooled and 1·5 ml. *N* HCl, 5 drops of starch and 1 ml. *N* KI are added, and the solution is titrated against *N*/500 thiosulphate using a 3 or 5 ml. micro-burette

$$1 \text{ ml. } N/500 = 26{\cdot}7\gamma \text{ bromine.}$$

The oxidation of the bromide in the presence of chloride is very conveniently carried out in the same flask as the titration so that there is very little chance of loss.

The preliminary heating on the water-bath with water and alkali causes partial if not nearly complete hydrolysis of the blood-proteins, and as the author has reason to think some of the bromine in the blood is bound to protein, there is less likelihood of loss and at the same time the use of huge quantities of alkali with consequent corrosion of dishes and basins is avoided. It also permits the use of nickel and porcelain basins for the ashing.

The chief aim in the ashing process is to evaporate most of the tarry matter at 400° and then to reduce the ash so that there is very little carbon left by heating at 500°. Grinding up the ash with water and igniting three successive times at 500° seems to remove any possibility of organic matter coming through to the hypochlorite stage. If organic matter is present it will lead to low figures. The method of ashing will not recover substances like bromobenzene or ethyl bromide which are immiscible and evaporate before fixation, but there is no reason to believe that such substances exist in blood.

The contents of the flask after the addition of the KClO must not be boiled as there is danger of excessive $KClO_3$ formation. The titration end-point is quite sharp, but owing to the large salt content of the solution the colour change is from blue-purple to colourless. The blank determination on the 10 ml. sucrose solution control which is put through exactly the same process is of the order of 0·4 ml. of *N*/500 thiosulphate and is due to a small amount of chlorate forming during the heating at 85° on the water-bath and includes the inevitable over-titration so that these both cancel out.

Iodides are also quantitatively oxidised by hypochlorite to iodates and are thus estimated together with bromide.

Normal blood, however, contains 500–1000γ/100 ml. bromine and 10–15γ/100 ml. iodine, and thus variations in the latter unless very large, as for instance after iodine therapy, do not appreciably affect the figures for bromine.

Table I. *Showing amounts of bromine as γ Br found in 10 ml. samples of normal human blood together with amounts found after bromide had been added.*

	Normal blood	+100 γ Br as KBr	Difference
A	89	184	95
B	120	217	97
C	173	277	104

SUMMARY.

A method is described for the determination of the bromine content of 10 ml. of normal blood, whether it contains 0·1 mg. or 100 mg./100 ml.

Through the courtesy of Sir Robert Robertson and the late Sir Walter Fletcher the author has had access to an unpublished report of work on the determination of iodine carried out for the Medical Research Council at the Government Laboratory by Mr C. O. Harvey, and he has been guided by it in choosing the conditions for the preliminary treatment and incineration of the blood and for the alcoholic extraction of the potassium bromide. He wishes to express grateful thanks for this privilege.

The author also wishes to thank Dr F. L. Golla, Director of the Central Pathological Laboratory, and Dr S. A. Mann for their advice and criticism. He is also indebted to the medical officers of the London County Mental Hospitals for their kindness in providing the samples.

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X. OBSERVATIONS ON THE ACTION OF CHLORINE ON LIGNIFIED TISSUES.

By FLORENCE MARY WOOD.

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(*Received November 28th, 1933.*)

During a study of the chemical nature of the cell membrane in plant tissues use was made of the chloramine reaction as a means of detecting protein in the cell-wall. Plant tissue sections were submitted to the prolonged action of chlorine gas and subsequently treated with aqueous solutions of sodium hydrogen phosphate and potassium iodide. Under these conditions proteins, if present, produce chloramines which liberate iodine from potassium iodide, resulting in a yellow coloration of the cell-wall [Wood, 1926].

This reaction led to interesting results with lignified tissues apart from its original application. A pink coloration was developed in these tissues [Wood, 1927], and while investigating the origin of this it was found necessary to devise some means of detecting vanillin in the cell-wall and of distinguishing this compound from coniferin, since it was thought possible that the coloration might be due to a compound of vanillin or coniferin, or their derivatives, with chlorine. Other possibilities were that it might be due to a compound of lignin with chlorine or to the nature of the lignin present. It has been shown by the author that this coloration is not due to oxidation of the cellulose base, but to the presence of the halogen [Wood, 1927].

Vanillin has been shown to occur in linden bark, in the summer only, by Bräutigam [1900, 1, 2]; it was found in a board by von Lippmann [1904], is stated to predominate in the oldest lignified tissues by Ellram [1896] and has been shown to occur in decayed oak-wood by Sullivan [1914].

It is well known that the sulphite and soda liquor from the manufacture of wood-pulp contains vanillin.

Coniferin occurs frequently in plant material. Kubel [1866] found it in the cambial sap of all conifers. It is converted by hydrolysis into coniferyl alcohol, and by subsequent or direct oxidation into vanillin [Tiemann and Harmann, 1874]. Hence it was necessary to perform distinguishing tests for these substances.

The possibility of the production of vanillin from coniferin, or on further oxidation vanillic acid by the chlorine-phosphate-iodide treatment to which the plant sections were submitted, must be taken into account, for Tiemann and Mendelsohn [1875] record the reverse process.

Should vanillin be detected after chlorination, its origin is a matter of some importance.

As the pink coloration only appeared in the presence of the halogen, and evidently a definite compound was formed, the problem was to show whether it was due to:

(*a*) a compound of chlorine with coniferin, or coniferyl alcohol which may be produced by the hydrolysis of coniferin;

(*b*) oxidation of coniferin to vanillin and the subsequent formation of a chlorovanillin;

(*c*) the formation of chlorovanillin from vanillin naturally present in the cell wall;

(*d*) the prolonged treatment with chlorine resulting in the formation of vanillic acid or possibly the production of a chlorovanillic acid.

No information could be obtained as to the existence of any compound of coniferin or coniferyl alcohol with chlorine, so that this part of the work was concerned with proving the presence or absence of the coniferyl nucleus before and after chlorination.

Menke and Bentley [1898] describe the formation of a chlorovanillin when vanillin in solution in chloroform is treated with chlorine for some time. Hann [1925] records the preparation of chlorovanillin, probably identical with the above, when vanillin dissolved in glacial acetic acid is treated with chlorine in the presence of fused sodium acetate.

But these chlorovanillins are colourless or yellow substances and the compound under investigation is pink, hence if chlorovanillin is produced, there must be some further reaction, resulting in a coloured compound.

Hann *et al.* [1929] describe the preparation of 5-chlorovanillal-3-nitro-*p*-toluidine in the form of iridescent brick-red granules. It should be noted that this possesses a deep colour because it is a nitro-anil; the colour is not associated with the presence of the chlorine.

They prepared also iodo-derivatives of substituted vanillins with the isomeric tolylrhodanic acids, and found that as a general rule all the iodo-derivatives are red, brown or deep yellow, whereas the analogous chlorine compounds are yellow. All these compounds were found to be insoluble in water, slightly soluble in alcohol and organic solvents, and to dissolve in concentrated sulphuric acid with the production of brilliant red colours. Hence there are two further possibilities of the origin of the observed pink colour; *viz.* it may be due to:

(*a*) the production of chlorovanillin and its subsequent union with an amine derived from the protoplasmic constituents of the cell, or from the cell-sap, to produce a chlorovanillal derivative which then reacts with the potassium iodide;

(*b*) to the formation of an iodo-derivative of the substituted vanillin on treatment with potassium iodide.

Experimental.

The experimental work was carried out on plant tissues, in the form of transverse sections of stems, and where possible similar experiments were performed on the chemical substances suspected. The twenty-three species examined included those which had been found already to give an intense pink coloration, those which gave a faint coloration and some which gave negative results. They included Gymnosperms, which all gave an intense coloration, and Angiosperms which gave a variety of results.

Owing to the comparatively small proportion of any section of the plant tissue giving the pink coloration in the chloramine reaction, no method has been devised so far for the extraction of the substance responsible, hence most of the work had to be based on colour reactions, although wherever possible these represented a definite chemical reaction and were not unexplained colour effects.

When bromine is used instead of chlorine a similarly coloured body is produced, though this element is not to be preferred to chlorine for use in this test [Wood, 1931].

The remote possibility of the metallic ion used having some effect also had to be considered.

A similar coloration to that produced when potassium iodide was used was obtained with sodium iodide and with magnesium iodide, it was therefore inferred that the nature of the metallic ion was without influence on the coloration. With potassium chloride, no pink coloration was obtained, and with potassium bromide, an orange-red coloration, which did not resemble the usual pink colour associated with this test, but was somewhat like that obtained when bromine was used instead of chlorine.

These results appear to give additional proof that the effect is connected with the presence of iodine.

In practice all results obtained were more satisfactory if the material, which was kept in alcohol, were placed in water for 12 to 24 hours before being submitted to the action of chlorine. The chlorinated material was then kept in alcohol (as otherwise, in spite of the presence of chlorine, fungi sometimes attack the sections) being treated with water again before completing the test.

Certain products of the chlorination of lignin dissolve in alcohol, *e.g.* lignone chloride [Cross and Bevan, 1895]. Although this substance is light yellow in colour, it was thought possible that it might react with iodine to produce a substance of a different colour. By the use of alcohol the lignone chloride is partially removed before proceeding with the rest of the test.

Schorger [1926] states that the yellow colour produced in lignified tissues by the action of chlorine is due to a combination of the chlorine with the lignin, and that if a dilute solution of sodium sulphite is applied, as in the Cross and Bevan method of determining cellulose, the liquid is coloured pink to purple-red [Bevan and Cross, 1880; Webster, 1883]. By this method the chlorinated products are stated to be removed from wood. Hence the next step was to remove such products of chlorination from the sections. This was effected by boiling them with 2 % sodium sulphite solution after chlorination, then treating them with sodium hydrogen phosphate and potassium iodide and comparing the colour reactions with those produced in sections which had not received the sodium sulphite treatment.

Transverse sections of the stems of fifteen species were examined in this way, chlorination being effected by chlorine gas and by chlorine dissolved in carbon tetrachloride. The pink colour appeared in the same way as if sodium sulphite had not been used, and there was only extremely slight coloration of the liquid.

Sections extracted with glacial acetic acid, which also dissolves lignone chloride, failed to give the test.

The last two series of experiments also show that the more satisfactory results obtained when material that has been preserved in alcohol is treated with water before and after chlorination should not be ascribed to the partial removal of lignone chloride, since the presence of this substance apparently has no effect upon the development of this pink coloration, although a certain amount of this substance is probably formed.

It was next necessary to show whether the pink colour was associated with the lignin complex or not; in investigating this point a large number of plants were examined. The reactions employed for lignin were those produced with diamine blue, brilliant Congo blue, orcinol, phloroglucinol, *o*-, *m*- and *p*-xylidene, and *m*-xylidene acetate.

In the majority of these reactions the pink colour was found to be associated with a positive reaction for lignin in primary and secondary xylem, and in many

cases in stelar sclerenchyma. The results obtained with cortical and stelar sclerenchyma were indefinite, pointing to considerable variation in the chemical composition of these tissues.

Since a connection had been established between the reaction and the lignin complex, the next step was to determine what parts or products of this complex were concerned. It was evident from the amount of the coloration, which in no case was so intense as to render the cell-wall opaque to light, that a portion only of the bulk of the cell-wall was concerned.

There are two types of lignin described by Paloheimo [1929], *ortho*lignin, which is resistant to the action of concentrated hydrochloric acid and is scarcely attacked by boiling with dilute alkali, and other kinds of lignin, called hydrolysable lignins, which are readily removed by strong acids and dilute alkalis.

Sections of the stems of seven species were treated separately with caustic soda and with hydrochloric acid and the aniline sulphate and Maule tests used to give the characteristic lignin reactions. The results were compared with those obtained from similar tests made on untreated material, the depth of colour indicating how much lignin had been removed.

All the above tests were performed both before and after chlorination and correlated with the chloramine test made on other sections of the same material. The pink colour is not influenced to any marked degree by the presence or absence of either *ortho*lignin or hydrolysable lignin.

The next possibility seemed to be that the colour was due to some compound either produced in small quantity from the lignin as a whole, or loosely associated with the lignin, or perhaps to some encrusting substance. As indicated earlier, two such substances are coniferin and vanillin. Differential experiments were made for the detection of coniferin and vanillin in plant-tissues, and the results obtained were correlated with those of similar experiments made with vanillin and coniferin themselves. These compounds were submitted to the action of chlorine gas, or to that of chlorine dissolved in carbon tetrachloride, and in every case to subsequent treatment with sodium hydrogen phosphate and potassium iodide. The colorations produced were mainly yellow to orange or brown with coniferin, and orange, red or reddish brown with vanillin, indicating that the latter was more likely to be associated with the coloration produced in plant tissues.

In general, the xylem formed in the autumn gave a stronger vanillin reaction than the spring xylem, which often indicated traces of coniferin. The older lignified tissues gave a stronger reaction for vanillin than the younger tissues, thus confirming Ellram's work [1896], and the younger tissues sometimes gave only a very slight reaction. This suggests that vanillin may be produced from coniferin as the tissues become older. Where there was a positive chloramine reaction, the colour reactions for vanillin were always more intense, particularly in two of the species examined, *viz. Laurus nobilis* and *Bambusa nigra*, and where there was a negative chloramine result, the reactions for vanillin were either negative or extremely slight.

As might be expected from the earlier work the reactions varied with the sclerenchymatous tissue involved [Wood, 1927]. The variation was mainly in the shade of the coloration indicating either vanillin or coniferin, the general result being that vanillin in varying amounts was indicated. The reactions for vanillin were generally stronger in cortical sclerenchyma than in stelar sclerenchyma.

The results obtained with all these tissues show that vanillin, and not coniferin, has some connection with the pink colour of the chloramine reaction.

This eliminates the possibility of it being due to a compound of chlorine with coniferin or coniferyl alcohol.

Since it was possible that coniferin, present in the cell-contents, or the cell-wall, might have undergone oxidation by the prolonged chlorine treatment, ultimately producing vanillin, the results of the previous experiments on material, both before and after chlorination, were carefully examined. Sections of six species were again submitted to the codeine test for vanillin [van Itallie and Steenhauer, 1927, 1, 2] both before and after chlorination. In most cases the vanillin appeared to be present before as well as after chlorination, showing that it was naturally present in the material and was not an oxidation product of chlorination.

The possibility of some of the reactions attributed to coniferin and vanillin being also given by hemicelluloses could not be ignored, particularly as these substances may be transition products in the change from pectic substance to lignin, when lignification of the cell-wall takes place [O'Dwyer, 1928].

Schultze defines hemicelluloses as the carbohydrates occurring in the plant-cell that are dissolved by dilute alkalis, readily hydrolysed by hot, dilute mineral acids and destroyed by Schulze's reagent [Schulze, 1890; 1891; 1892]. Schorger [1926] in addition considers that they should be soluble in boiling water.

Hemicelluloses, such as wood gum or xylan, may be present in the cell wall. In order to determine whether wood gum or xylan were present in the original material used, the following experiments were carried out with sections of stems of eight species. Some sections were treated with 5 % aqueous caustic soda in the cold and the usual phloroglucinol test applied, both in the cold and on warming.

The latter test was also made on untreated material both in the cold and on warming. The xylan is removed by caustic soda. Xylan is coloured by phloroglucinol on warming, while lignin is coloured in the cold [Wheeler and Tollens, 1889]. If a positive reaction is produced on the untreated material in the cold this is due to lignin alone, but the coloration produced on warming is due to the combined action of lignin and xylan. Naturally if xylan is removed with caustic soda, any coloration is due to lignin alone. Maximum effect was produced if the sections, which had been treated with caustic soda, were left in water for some days before being treated with phloroglucinol.

The colour produced with phloroglucinol was even more brilliant when the sections were treated with caustic soda, which seems to show that in these specimens there was not any appreciable amount of xylan and certainly not enough to have any marked effect in the tests with which this paper is concerned.

The colour produced with phloroglucinol was examined carefully in every case for any reddish-violet tinge, as this indicates the presence of a compound of *iso*-eugenol with oxygen [Herzog and Hillmer, 1929]. Dilute alkalis produce *iso*-eugenol from eugenol, and this on oxidation gives vanillin.

Only in two of the Gymnosperms, *Cycas revoluta* and *Larix europaea* was any purplish tinge perceptible in untreated material in the cold and on warming. In *Larix europaea* when the material had been treated with caustic soda this coloration persisted, but only when the phloroglucinol test was made in the cold. This shows that *iso*-eugenol was not usually present in the material used in these tests.

Caustic soda removes any coniferyl alcohol present, so that none of the above results, after soda treatment, can be attributed to this substance.

The possibility of the connection of vanillin and chlorine with the development of the pink colour obtained in the chloramine reaction having been

established the nature of the compound present was next investigated. Further confirmation of the responsibility of the halogen was afforded by the analogous results which were obtained when bromine was substituted for chlorine [Wood, 1931]. Chlorovanillin, at first sight, appears to be an obvious solution, but this substance is colourless or light yellow in colour, so, should the suspected connection be true, the compound responsible for the coloration must be a product of the reaction of chlorovanillin with either (*a*) the other reagents used, (*b*) some substance occurring in the cell or cell-wall, or (*c*) with both the former and the latter.

Chlorovanillin was prepared according to Hann's method [1925], and experiments were made with it in the endeavour to clear up these points. In the first place, the reagents used, *viz.* sodium hydrogen phosphate and potassium iodide, were investigated.

Chlorovanillin was used (*a*) dissolved in alcohol, (*b*) in hot water, alone and in the presence of acetic acid, and separate experiments were carried out with each of these four solutions, using:

(1) sodium hydrogen phosphate and potassium iodide;

(2) sodium hydrogen phosphate;

(3) potassium iodide in which iodine had been dissolved, to obtain the maximum effect of the iodine which may be set free by the chloramine produced from any protein present in the cell-contents.

In no case was there any indication of a pink coloration, hence chlorovanillin and the reagents used are not alone responsible for this pink colour.

Since 5-chlorovanillin can form vanillal derivatives with amines (see above), the next experiments involved the examination of the effect of sodium hydrogen phosphate and potassium iodide upon:

(*a*) chlorovanillin and an amine;
(*b*) vanillin and a chloramine;
(*c*) chlorovanillin and a chloramine.

The slight acidity due to the sodium hydrogen phosphate must be taken into account when considering the results obtained, hence a similar series of experiments was conducted in which the phosphate was omitted.

Further, since the possibility of the effect being due to a substituted compound of vanillin had to be considered, the whole of this part of the work was repeated substituting vanillic acid for vanillin and vanillic acid treated with chlorine for chlorovanillin.

The experiments were conducted using:

(*a*) sodium hydrogen phosphate and potassium iodide;
(*b*) sodium hydrogen phosphate alone;
(*c*) potassium iodide alone, on all the substances enumerated above. All the solutions used were alcoholic, ethyl alcohol being employed. Sodium hydrogen phosphate and potassium iodide were used in the solid form.

The compounds containing chlorine and an amine group used were chloramine-T, *p*-chloroaniline, *o*-chloroaniline, and the compounds containing the amine group alone were dimethylaniline, dimethylaniline hydrochloride, aniline hydrochloride and xylidine acetate. In the first series of experiments the method of procedure was to add the amine to the vanillin in a test-tube, boil and then to add a little solid sodium hydrogen phosphate and potassium iodide, then a few drops of water, just sufficient to effect the solution of these substances, and to boil again. The resulting liquid was then put into small porcelain dishes and a

strip of filter-paper immersed in it, with one end out of the liquid, to observe the effect of the mixture upon cellulose, the object being to simulate an encrusting substance upon a cellulose base as might occur in the plant cell-wall.

Slow evaporation to dryness in the air was allowed to take place and the effect on the filter-paper carefully watched. As one end of the filter-paper was arranged to be out of the liquid, the colour effect produced could be observed as the liquid crept up the paper and then slowly evaporated. By arranging the amount of liquid in each dish carefully, the free end of the filter-paper was kept dry at the top. This was necessary, as many of the colour effects produced appeared as the paper dried just where the wet paper met the dry paper. The second and third series of experiments were conducted in a similar way, except that in the second series the potassium iodide was omitted, and in the third series no sodium hydrogen phosphate was used.

The three series of experiments were repeated using a strip of superfine "Tarantulle" instead of the filter-paper.

Finally, the whole of the experiments were repeated, as a confirmation of the results obtained.

In the latter series of experiments in addition to the filter-paper and superfine "Tarantulle", strips of purified wood-pulp were used.

Vanillin produced a pink colour on these materials when used with *p*-chloroaniline and *o*-chloroaniline in the presence of potassium iodide, the most brilliant effect being produced when both the sodium hydrogen phosphate and the potassium iodide were employed. Chlorovanillin produced a similar pink colour with *p*-chloroaniline and *o*-chloroaniline when potassium iodide was present alone and also when sodium hydrogen phosphate was used as well.

Vanillic acid gave a slight pink coloration in the presence of *o*- and *p*-chloroaniline respectively when the iodide was present. It is possible that this may be due to minute traces of vanillin in the vanillic acid.

Vanillic acid treated with chlorine gave indefinite results, owing to the liberation of iodine, which obscured any other colorations that might have occurred.

There was some indication of a faint pink coloration with both *o*- and *p*-chloroaniline, possibly due as indicated above to minute traces of vanillin.

In the last series of experiments it was found that frequently a considerable time had to elapse, often as much as twelve hours, before the colour developed, so that in every case the strips of material were examined the following day, in addition to the preliminary examination. This was probably due partly to variation in the atmospheric saturation and partly to variations in temperature which altered the rate at which evaporation of the liquid took place. This evaporation could not be increased by gentle warming as then either the colour did not appear at all, or it appeared only momentarily and rapidly disappeared.

In no case was it a permanent coloration, fading even at the ordinary temperature in the course of a few days.

Similar effects have been observed in sections of plant material which developed a pink coloration in the xylem after being submitted to the chloramine test which has already been described. In these cases also this coloration takes a certain time to develop and fades in the course of a few hours.

As additional proof that chlorine is an element essential to the reaction, and as a control to the other work, another series of experiments was made, using strips of filter-paper, "Tarantulle" and wood-pulp as before, and employing vanillin and vanillic acid respectively, with dimethylaniline hydrochloride and aniline hydrochloride and so having no chlorine present either substituted in the

vanillin nucleus or in the amino compound. These experiments were entirely negative.

Hence the results indicate that:

(1) potassium iodide (*i.e.* iodine) is essential to the reaction;

(2) the presence of sodium hydrogen phosphate is without effect upon the reaction;

(3) an amine and chlorine must be present.

The fact that an amine is essential to produce this coloration eliminates the possibility of its being due to an iodo-derivative of the substituted vanillin.

The conclusion that must be drawn is that in these experiments most probably some kind of vanillal derivative is produced from the vanillin, chlorine and amine, similar to those prepared by Hann *et al.* [1929] and that it reacts with the iodine to produce a pink-coloured substance, though whether the compound is an additive or substituted one remains to be determined. The method of experiment introduces many variables including atmospheric oxidation of the amines, but it was employed because it was to similar conditions that plant sections were exposed during the experiments made upon them.

To elucidate the reactions that take place in plant material is a difficult matter, but with reference to the pink coloration produced in the chloramine reaction when employed with such material the following facts have been proved:

(*a*) it is associated with vanillin and chlorine;

(*b*) it only appears when iodine is subsequently present.

Although the cell-wall does not contain any appreciable amount of protein [Wood, 1926], its close association with the cell-contents, which are rich in amino-compounds, renders these available for possible reactions. The experiments with chemical substances indicate that colorations are produced, when the conditions are such that a vanillal derivative might be formed, which, reacting with iodine, produces a substance resembling in colour and properties that appearing in the lignified tissues of some plants when treated with chlorine and subsequently sodium hydrogen phosphate and potassium iodide.

The inference drawn is that most probably in this reaction chlorine acts upon the vanillin in the cell-wall, forming chlorovanillin which reacts with a chloramine or mixture of chloramines, produced from protein within the cell to produce a vanillal, which gives a coloured substance when iodine is present.

Should the chlorine not affect the vanillin to any marked degree, probably vanillin itself may react with the chloramines from the cell-contents and still produce the coloration.

It is possible that both series of reactions take place simultaneously.

Having found some indication of the kind of substance producing this coloration, and the chemical experiments with filter-paper, "Tarantulle" and wood-pulp showing that it is possible that the vanillin responsible is absorbed or at least very loosely united with some form of lignin as a base, experiments are now being carried out to investigate these points and the chemical nature of the base.

Since it has been found that there is more vanillin in older than in younger tissues, and more in autumn than in spring xylem, the author suggests that this substance may be present as an excretory product, which is set free when the function of the tissues becomes more largely that of mechanical support. This upholds the theory that has been advanced that the vanillin present is either adsorbed upon the lignin base or only loosely associated with this base.

SUMMARY.

1. When investigating the origin of a pink colour produced in lignified tissues when the chloramine reaction is employed, differential tests for coniferin and vanillin were made.

2. Experimental work is described in which it is shown that the coloration is not due to lignone chloride or to the products of chlorination removable by sodium sulphite, that it is closely associated with the presence of iodine and also of lignin, but that the nature of the lignin itself is not concerned, so that the latter association is either very loose or the substance is encrusted on the lignin. It is shown that vanillin is present when the coloration is produced, and that there is more vanillin in older than younger tissues, in autumn than spring wood, in cortical than in stelar sclerenchyma, and that it is naturally present in the cell-wall. Hemicelluloses and coniferyl alcohol are not responsible for the observed effect.

3. Under similar conditions to those used with plant sections experiments were made with vanillin, chlorovanillin, vanillic acid, vanillic acid treated with chlorine, and amines or chloramines, in which filter-paper, "Tarantulle" and wood-pulp were used upon which to "encrust" the compounds concerned. Indications were obtained that a vanillal derivative was formed which reacted with iodine to produce a similarly coloured substance to that obtained in plant tissues.

4. The conclusion drawn is that vanillin, in loose association with some form of lignocellulose, is acted upon by chlorine producing a chlorovanillin, which with the chloramines produced from protein within the cell, forms a vanillal derivative which gives a pink-coloured substance in the presence of iodine.

5. The possibility that vanillin is an excretory product, produced when the function of the tissues concerned has become chiefly that of mechanical support, is discussed. This theory is upheld by the fact that vanillin predominates in older tissues and in autumn wood.

These investigations were carried out in the Botanical department of Birkbeck College, University of London, and have been assisted by a grant from the Royal Society and another from the Dixon Fund of the University of London. The author desires to express her thanks to Prof. Dame Helen Gwynne Vaughan for valuable help and advice during the progress of the work and to Prof. Sugden for many helpful criticisms and suggestions.

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XI. URICASE AND ITS ACTION.
VII. SOLUBLE URICASE.

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(*Received December 8th, 1933.*)

It has been shown in Parts II, III and IV [Truszkowski, 1930] of this series that tissue uricase is associated exclusively with the insoluble fraction of the tissues, and that it is not extractable by aqueous solutions of p_H 6–11, by aqueous glycerol or by other agents generally used for extraction of enzymes.

Ro [1931] described a method of extracting uricase from tissues similar in principle to that of Wiechowski and Wiener [1907], whereby he obtained extremely active extracts. Ro's method consists in the acetone precipitation of an aqueous extract of ox-kidney followed by repeated treatment of the precipitate with acetone, alcohol and ether. The white powder thus obtained yields uricolytic solutions on extraction during an unspecified time with 0·125 % Na_2CO_3 solution.

It was found by the present author that the dry preparation prepared in the above manner yielded active filtrates after extraction during 24 or 48 hrs. at 37° with Na_2CO_3.

The possibility of obtaining soluble uricase by Ro's method having thus been confirmed, the necessity arose of reconciling this finding with the negative results previously obtained. The differences between the two methods consisted in the use of dry, lipoid-free kidney tissue by Ro, in the presence of water-soluble kidney constituents, and in the use of Na_2CO_3 solution for extraction, instead of the glycine-NaOH buffers used by the present author. With regard to the first point, it seemed improbable that the extractability of uricase could be affected by merely drying the tissues; the necessity of the alcohol-ether treatment should lie rather in removal from the tissues of some substance, probably lipoid, which prevents extraction from fresh tissues. As to the second point, it has been shown (Part IV) that the water-soluble constituents are inactive in the fresh state; experiments described below show that the same applies to this fraction after treatment according to Ro's method. Finally, extractions performed on fresh kidney tissue with aqueous Na_2CO_3 were found to yield inactive filtrates.

Experimental.

Ox-kidney uricase was prepared by the method described in Part IV of this series. Uric acid was dissolved in boiling aqueous NaOH (65 ml. 0·1 *N* NaOH per g. uric acid); the solutions so prepared had p_H 7·5–7·8. Uric acid was determined by the method of Folin and Denis.

1. *Conditions of extraction.*

Since Ro's paper gives no details as to the duration and temperature of extraction, the first step was to determine these conditions. 1 g. of dry product prepared according to his method was suspended in 100 ml. of 0·125 % Na_2CO_3,

1 ml. of toluene was added to ensure sterility, and the activity of the extract was determined after standing 1, 24 and 48 hrs. at 35° by adding 5 ml. of filtered extract to 10 ml. of 0·144 % sodium urate, with 1 ml. of toluene, and determining uric acid after incubation at 35° during 18 hrs. No diminution in uric acid content was observed in 1 hr. extracts, 24 hr. extracts oxidised 6·1 mg., and 48 hr. extracts oxidised 7·2 mg. of uric acid. The residue left after extraction still contains extractable uricase, as was shown by the following experiment. 1 g. of dry uricase was extracted during 48 hrs. at 35° with 100 ml. of 0·125 % Na_2CO_3, filtered, the extract stored in a stoppered flask with toluene and the residue extracted as before with a fresh portion of Na_2CO_3, the second residue being again extracted. 5 ml. of the first extract and 5 ml. of Na_2CO_3 solution, and 10 ml. of the second and third, were left at 35° during 18 hrs. with 10 ml. of 0·13 % uric acid, after which uric acid was determined. 6·3 mg. of uric acid had been oxidised by the first extract, 5·4 mg. by the second and 1·0 mg. by the third, corresponding with an activity of 1·26 mg. per ml. of uricase in the first case, 0·54 mg. in the second and 0·1 mg. in the third.

2. *Comparison of activity of products and extracts.*

Hashed kidney tissue was shaken with 10 volumes of water, the suspension was filtered through cheese-cloth, and a part of the filtrate was treated according to Ro. The remainder was centrifuged, the centrifugate was filtered and the filtrate treated with acetone, *etc.*, by Ro's method; the residue was shaken successively twice with alcohol and twice with ether and then air-dried at 30°. The various products thus obtained were extracted with 0·125 % Na_2CO_3, and the activity of the extracts and the suspensions in 0·125 % Na_2CO_3 was determined.

The products examined were: Exp. 1, 1 % suspension of residue (I) obtained after centrifuging the suspension (III) of kidney tissue; Exp. 2, the

Table I. *Comparison of activity of products. All flasks contain 10 ml. of 0·104 % sodium urate and 10 ml. of uricolytic suspension, extract or 0·125 % Na_2CO_3.*

		mg. uric acid			
		After 18 hrs. at 35°		After 42 hrs. at 35°	
No. of Exp.	Product examined	Found	Oxidised	Found	Oxidised
Control	0·125 % Na_2CO_3.	10·4	—	10·4	—
1	1 % suspension of untreated uricase (I).	0·4	10·0	0	10·4
2	Filtered centrifugate (II) of tissue suspension (III).	10·2	0·2	8·2	2·2
3	1 % suspension of dry product (IV) obtained from (II).	10·0	0·4	9·2	1·2
4	0·17 % suspension of dry product (V) obtained from (I).	1·4	9·0	0	10·4
5	0·17 % suspension of dry product (VI) obtained from (III).	1·2	9·2	0	10·4
6	1 % extract of (IV).	10·4	0	10·4	0
7	„ „ „ (V).	0·6	9·8	0	10·4
8	„ „ „ (VI).	0·6	9·8	0	10·4
9	6 % „ „ (I).	10·0	0·4	9·6	0·8

filtered centrifugate (II); Exp. 3, a 1 % suspension of the dry product (IV) obtained by treating (II) according to Ro; Exp. 4, a 0·17 % suspension of the product (V) obtained by extracting 20 g. of (I) successively with two 200 ml. portions of 96 % alcohol, and with two 100 ml. portions of ether and drying at 35°; Exp. 5, a 0·17 % suspension of the dry product (VI) obtained by Ro's procedure; Exp. 6, 7 and 8, 1 % 24 hr. extracts of (IV), (V) and (VI); Exp. 9, a 6 % extract of (I). 1 % suspensions of (I) were compared with 0·17 % suspensions of (V) and (VI) in view of the fact that the dry content of (I) was about 15 %, as compared with 95 % in (V) and (VI).

It follows from the results obtained (Table I) that active products are obtained exclusively from material containing (I); *i.e.* uricolytic activity is associated exclusively with the insoluble parts of kidney tissue. The slight activity shown by the centrifugate and by extracts of (I) is ascribable to very fine, filter-passing particles of (I). A comparison of Exps. 1, 4 and 5 shows that the activity of (I) is very little affected by treatment with acetone, alcohol, ether and by drying. A comparison of Exps. 1, 7 and 8 shows that only a small part of the original activity of (I) is exhibited by the extracts, since the content of anhydrous material taken for extraction was 6 times greater in Exps. 7 and 8 than that present in Exp. 1. Exps. 4 and 5, 7 and 8 indicate that the uricolytic properties of Ro's product are practically identical with those obtained by treating (I) with alcohol and ether. Finally, Exp. 9 shows that uricolytic extracts cannot be obtained from (I) under conditions comparable with those of extraction of (V) and (VI).

3. *Simplified method of preparation of soluble uricase.*

The experiments described in the preceding section indicate that Ro's technique is unnecessarily complicated and results probably in the inclusion of a certain proportion of inactive matter in his dry products. The steps which appear to be superfluous are: 1. allowing the kidney-tissue suspension to stand with thymol; 2. treating the suspension with acetone. The following procedure was adopted in place of Ro's. Uricase is prepared as described in Part IV of this series, shaken during 2 mins. with 10 vols. of 96 % alcohol and rapidly filtered by suction; the process is repeated once more with alcohol and twice with the same volume of ether, after which the product is allowed to dry at room temperature, or at 37°. It is important that residual ether be allowed to evaporate spontaneously, without expressing on filter-paper, as the product obtained in the latter case is lumpy, black and yields relatively inactive extracts. The final product (this will in future be designated "dry uricase") is a white, impalpable powder, containing 5 % of moisture, and can be stored indefinitely. The yields of dry uricase average about 10 g. per kg. of kidney, as compared with 100 g. obtained by Ro. It is difficult to understand how such high yields could have been obtained, as the average dry content of ox-kidneys does not exceed 15–20 %, and by far the greater part of the raw material remains unsuspended. Moreover, of the solid matter of the suspension filterable through muslin, about 10 % is represented by lipoids, removed during the alcohol-ether extraction, and a further 10 % is present as water-soluble extractives. It must therefore be considered that, whilst somewhat higher yields than those obtained by the present author might be obtained by finer comminution of the original tissues, yet in no case could yields much higher than 1·5 % of dry uricase be expected without considerable contamination by inert matter. Solutions of uricase are obtained under the conditions described in section 1 of this paper.

4. *Significance of lipoid extractives.*

The combined alcoholic extracts from 100 g. of uricase were evaporated to dryness under reduced pressure at 38°, when about 3 g. of a semi-solid, oily, yellow residue, completely soluble in chloroform, were obtained. The combined ethereal extracts, similarly treated, yielded about 2 g. of a dry, white residue, M.P. 52–54°, also soluble in chloroform; 1 g. of each residue was dissolved in 100 ml. of ether, and 1 g. in chloroform. Three 0·2 g. portions of dry uricase were introduced into 100 ml. Erlenmeyer flasks, 5 ml. of the respective ethereal solutions or ether alone were added, and the ether was removed under reduced pressure at $\not> 37°$. 20 ml. of 0·125 % Na_2CO_3 and 1 ml. of toluene were then added to each flask, their contents were filtered after 48 hrs., and the uricolytic activity of the extracts was determined as usual, taking 10 ml. of Na urate and 2 ml. of filtrate.

Table II.

No. of Exp.	Solution added to dry uricase	mg. uric acid: After 18 hrs. at 37° Found	After 18 hrs. at 37° Oxidised	After 66 hrs. at 37° Found	After 66 hrs. at 37° Oxidised	
1	5 ml. ether	3·4	7·4	0	10·8	
2	5 ml. ethereal solution of alcoholic extractives	10·8	0	10·8	0	
3	5 ml. ethereal solution of ethereal extractives	10·8	0	10·8	0	
4	—	10·8	—	10·8	—	Control : 10 ml. sodium urate, 2 ml. 0·125 % Na_2CO_3.

The results obtained (Table II) indicate that the extractives removed by alcohol and by ether prevent extraction of uricase by aqueous Na_2CO_3, *i.e.* uricase is fixed on the tissues by the agency of lipoid substances (Exps. 2 and 3); Exp. 1 shows that the results obtained are not ascribable to inactivation of uricase by ether.

This conclusion as to the rôle of lipoids in the systems in question was further established by the following experiments. Three 0·5 g. portions of dry uricase were placed in three 100 ml. flasks; to one, 5 ml. of a 1 : 1 mixture of the chloroform solutions of the alcoholic and ethereal residues were added, to another

Table III.

No. of Exp.	Solution added to dry uricase	mg. uric acid after 24 hrs. at 37° Found	Oxidised	Remarks
1	5 ml. $CHCl_3$	0·2	9·0	
2	,,	0·2	9·0	Lipoids added to filtrate
3	5 ml. $CHCl_3$ solution of combined EtOH–Et_2O extractives	8·2	1·0	
4	5 ml. $CHCl_3$ solution cont. 5 mg. cholesterol	2·6	6·6	
5	—	9·2	—	Control : 10 ml. sodium urate, 10 ml. 0·125 % Na_2CO_3

5 ml. of chloroform containing 5 mg. of cholesterol, and to the third 5 ml. of chloroform. As before the solvent was evaporated at 37°, and the dry residues were extracted by 25 ml. of 0·125 % Na_2CO_3 during 48 hrs. at 37°. 10 ml. of each extract and 1 ml. of chloroform were then added to 10 ml. of sodium urate, and uric acid was determined after 24 hrs. at 37°; in addition, to a second 10 ml. portion of the extract of uricase to which lipoids had not been added, 1 ml. of the combined chloroform solution was added instead of pure chloroform.

The above experiments (Table III) confirm those of Table II; the slight activity shown by the extract in Exp. 3 as compared with the analogous experiment of Table II is probably ascribable to the greater concentration of uricase (10 times greater). Exp. 4 shows that the action of tissue lipoids is not necessarily specific, as the activity of the extract is reduced by 27 % by 5 mg. of cholesterol (50 mg. of lipoids were present in Exp. 3). Finally Exp. 2 proves that the lipoids do not inactivate extracted uricase, *i.e.* that their action consists in preventing extraction and not in inactivating the enzyme.

5. *Properties of soluble uricase.*

The extracts of soluble uricase are clear or slightly opalescent, straw-coloured liquids, possessing an odour similar to that of aliphatic amines and foaming on shaking. On keeping, they become turbid but do not lose their activity. Quite definite protein reactions are obtained (Millon, Adamkiewicz, biuret), and the N content amounts to 0·08–0·13 %. The p_H of the extracts is invariably less than that of the original Na_2CO_3 solution (p_H 9·12).

A preliminary survey of the properties of soluble uricase was made, preparatory to more systematic work. The first problem was that of further purification by means of filtration. A suspension of 1 g. of dry uricase in 100 ml. of aqueous Na_2CO_3 was allowed to stand during 24 hrs. at 35°, and 10 ml. of the well mixed suspension (I) were measured into a 100 ml. flask. The remainder of (I) was then filtered through filter-paper, 10 ml. of the filtrate were taken for examination, and the remainder was filtered through a Berkefeld filter; finally, a portion of this filtrate (III) was passed through a Bechhold ultra-filter (filtrate IV). 10 ml. of sodium urate solution and 1 ml. of toluene were added to 10 ml. of the various preparations examined, and uric acid was determined after 18 hrs. at 35°.

Table IV.

No. of Exp.	Nature of preparation	mg. uric acid after 18 hrs. at 35°	
		Found	Oxidised
1	Control	14·4	—
2	Suspension	0	14·4
3	Filtrate (II)	6·6	7·8
4	Berkefeld filtrate (III)	7·0	7·4
5	ditto, 24 hrs. later	7·0	7·4
6	ditto, refiltered	9·0	5·4
7	ditto, 4 days later	9·2	5·2
8	Bechhold filtrate (IV)	9·5	4·9

Filtrates (III) and (IV) were water-clear but (III) became turbid on standing during 24 hrs. at room temperature, without, however, losing any of its activity (Table IV, Exp. 5). Part of the activity is in the precipitate, as is shown by Exp. 6, in which the Berkefeld filtrate of the turbid extract was examined. The clear, second filtrate again became turbid on keeping a further 4 days, with slight inactivation (Exp. 7). The Bechhold ultra-filtrate, obtained using a membrane impermeable to ovalbumin, exhibited moderate activity (Exp. 8). Protein reactions were given by all filtrates, in decreasing order of intensity.

6. *Action of ammonium chloride and of methylamine hydrochloride on uric acid.*

In view of Schittenhelm and Chrometzka's [1927] contention that ammonia acts uricolytically, and of the ammoniacal odour of the extracts, it was thought desirable to investigate the possibility that the activity of the latter might be due to ammonia or methylamine. With this object, a number of systems were set up, consisting of 10 ml. of 0·1 % sodium urate, 10 ml. of 0·125 % Na_2CO_3, and 10 ml. of 0·08 % NH_4Cl, or of 0·02 %, 0·04 % or 1 % $CH_3.NH_2.HCl$. The uric acid content, determined after 24 hrs. and 48 hrs. at 37°, remained constant, pointing to the non-identity of uricase with these substances.

Discussion.

From the biological point of view the most interesting phenomenon of those described above is that of the action of lipoids in preventing extraction of uricase. Whilst it would be premature to enter into a detailed consideration of the mode of action of lipoids in this connection, yet it appears from a number of investigations now in progress in this laboratory that this binding action of lipoids is of considerably wider incidence than in the immobilisation of enzymes on tissues, evidence of its presence having been found in widely different biological processes. For the purposes of this paper, however, the conclusion may be drawn that biologically uricase acts as an insoluble enzyme.

The preliminary survey of the properties of soluble uricase confirms Ro's finding that uricolytic activity is indissociable from traces of alkali-soluble protein. This protein is of relatively small molecular weight, as it passes freely through an unglazed porcelain filter and partly through a membrane impervious to ovalbumin. Further work on soluble uricase will be described in the succeeding parts of this series.

Summary.

1. Ro's method [1931] for the preparation of soluble uricase has been investigated, and his results have been substantially confirmed.

2. The non-extractability of uricase from fresh tissues is attributable to the presence of lipoids; after their removal by the action successively of alcohol and ether, uricase may be extracted by dilute aqueous Na_2CO_3.

3. A consequence of the above finding is that uricase acts *in vivo* as a contact catalyst.

4. Dissolved uricase passes freely through Berkefeld filters, and partly through Bechhold filters, the membrane of which is impermeable to ovalbumin.

5. Uricolytic activity varies roughly in proportion to the intensity of the protein reactions of the solution.

6. Ammonium chloride and methylamine hydrochloride have no uricolytic action; soluble uricase is therefore not identical with these substances.

The author wishes to express his gratitude to Prof. St J. Przyłęcki for his helpful suggestions and criticism during the conduct of the above research.

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XII. THE CONFIGURATIVE RELATIONSHIP OF THYROXINE AND TYROSINE.

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(*Received December 9th, 1933.*)

Since the constitution of thyroxine has been known it has been generally assumed that the compound is formed in the organism by the oxidative coupling of two molecules of 3 : 5-diiodotyrosine. The likelihood of such a reaction was indeed already referred to by Harington and Barger [1927] in the consideration of the then unknown orientation of the iodine atoms in the thyroxine molecule, and the hypothesis was considerably strengthened by the demonstration by Harington and Randall [1929] of the actual occurrence of 3 : 5-diiodotyrosine side by side with thyroxine in the thyroid gland.

Search which has been made in this and other laboratories for the possible existence of an enzymic mechanism capable of converting 3 : 5-diiodotyrosine into thyroxine has hitherto proved fruitless, so that direct biochemical evidence of the relationship of the two compounds is still lacking; nor is the hypothesis as yet susceptible of proof by biological means. There remains however a line of chemical investigation which is described in the present paper and of which the results appear to be conclusive.

It is evident that if thyroxine is in fact synthesised from 3 : 5-diiodotyrosine under biological conditions it must be configuratively related to the latter amino-acid and therefore also to tyrosine from which diiodotyrosine is undoubtedly derived. A chemical synthesis of thyroxine from natural *l*-tyrosine conducted by a series of reactions involving no racemisation and no possibility of a Walden inversion at any stage should lead therefore to the naturally occurring modification of thyroxine.

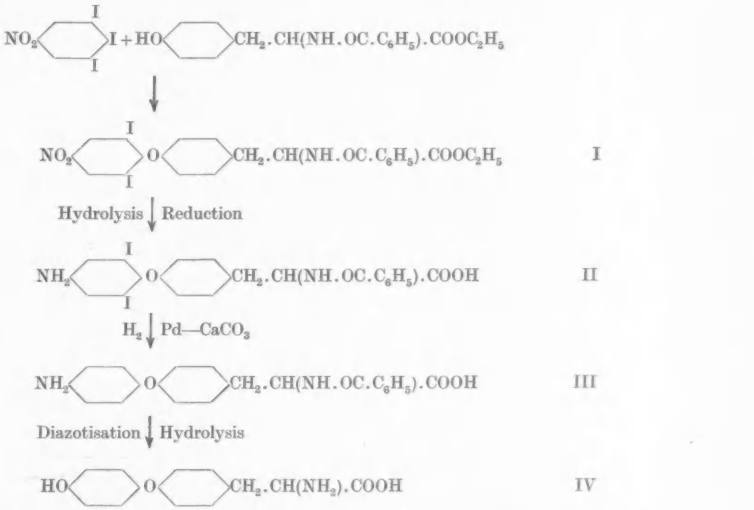
Unfortunately a direct synthesis of thyroxine itself from tyrosine or 3 : 5-diiodotyrosine is not feasible; on the one hand the halogenation of the thyronine nucleus in the 3 : 5-positions cannot be accomplished and on the other it is impossible to carry out the phenyl ether condensation with a phenolic substance such as 3 : 5-diiodotyrosine which is substituted in the *ortho*-positions to the hydroxyl group.

The problem has therefore had to be solved in an indirect manner. By a series of reactions to be described below it has been possible, starting from natural *l*-tyrosine, to synthesise an optically active thyronine having $[\alpha]_{5461}$ $+13{\cdot}3°$. Now we had in our hands some *l*-thyroxine which had been prepared by the resolution of *dl*-thyroxine according to Harington [1928] and which had been shown by the work of Harington and Salter [1930] to be identical with the naturally occurring compound; catalytic deiodination of a sample of this *l*-thyroxine yielded a preparation of thyronine having $[\alpha]_{5461} + 12{\cdot}2°$.

There can thus be no longer any doubt of the stereochemical relationship of natural *l*-thyroxine with natural *l*-tyrosine, and on chemical grounds at least we may feel secure in advancing the hypothesis that in nature thyroxine is derived from tyrosine.

Synthesis of d-*thyronine.*

The actual synthesis of *d*-thyronine from *l*-tyrosine was carried out as follows. The ethyl ester of *N*-benzoyl-*l*-tyrosine was condensed with 3 : 4 : 5-triiodonitrobenzene by boiling in methylethylketone solution with potassium carbonate to give ethyl α-benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]-propionate (I). (The object of employing 3 : 4 : 5-triiodonitrobenzene instead of the more



obvious *p*-iodonitrobenzene is simply to improve the yield at the stage of the condensation; the subsequent removal of the 3′ : 5′-iodine atoms offers no difficulties.) The ester (I) was hydrolysed to the free acid, which in turn was reduced with ferrous sulphate and baryta to the corresponding amino-compound (II); the latter was deiodinated by catalytic hydrogenation in presence of palladised calcium carbonate, and the resulting iodine-free amino-acid (III) was diazotised in dilute hydrochloric acid solution. The *N*-benzoylthyronine, which resulted on boiling the solution of the diazonium salt, was not isolated but was converted directly into *d*-thyronine (IV) by further boiling after the concentration of hydrochloric acid had been increased to 5*N*.

As already mentioned the thyronine obtained in this way had $[\alpha]_{5461} + 13{\cdot}3°$, whilst a sample of thyronine obtained by the catalytic deiodination of *l*-thyroxine ($[\alpha]_{5461} - 3{\cdot}5°$) had $[\alpha]_{5461} + 12{\cdot}2°$; a third sample obtained by deiodination of *l*-3 : 5-diiodothyronine (itself related to *l*-thyroxine) had $[\alpha]_{5461} + 13{\cdot}9°$.

Experimental.

N-*benzoyl*-l-*tyrosine ethyl ester.* This compound was most conveniently prepared as follows. *l*-Tyrosine ester hydrochloride (24·5 g.) was dissolved in the minimum amount of water; chloroform (200 ml.) was then added and the

mixture cooled to $-10°$. The free ester was liberated by addition of $5N$ sodium hydroxide (20 ml.) with vigorous shaking. Benzoyl chloride (14 g.) was then added in portions with shaking after each addition. The mixture was treated with $2N$ sodium carbonate (50 ml.) followed by a further equal amount of benzoyl chloride in portions. When evolution of CO_2 no longer occurred on shaking, the chloroform layer was separated, washed with water, dried over calcium chloride and evaporated; recrystallisation of the residue from a mixture of chloroform and light petroleum afforded 85 % of the theoretical amount of a product having M.P. 119°.

Ethyl α-benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]propionate (I). *N*-benzoyl-*l*-tyrosine ethyl ester (20 g. : 15 % excess) and 3 : 4 : 5-triiodonitrobenzene (28 g.) were dissolved in methylethylketone (200 ml.); freshly dried and finely powdered potassium carbonate (8·7 g.) was added and the mixture boiled under reflux for 18 hours. The solution was filtered and the precipitate extracted with hot methylethylketone; the combined filtrates were evaporated on the water-bath to a pasty residue which was triturated with cold water. The insoluble portion which was somewhat sticky was collected and recrystallised from 95 % alcohol (1800 ml.). The yield was 17·2 g. (45 % of the theoretical).

After recrystallisation from acetic acid the compound formed colourless prismatic needles, M.P. 189°. It had $[\alpha]_{5461} - 1{\cdot}8°$ ($c = 3{\cdot}65$ in acetic acid; 2 dm. tube). (Found: N, 4·0; I, 37·3 %. $C_{24}H_{20}O_6N_2I_2$ requires: N, 4·1; I, 37·0 %.)

α-Benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]propionic acid. The above ester (16 g.) was suspended in alcohol (55 ml.); 5·1N sodium hydroxide (5 ml.) was added and the mixture warmed on the water-bath with constant stirring; it became at first reddish in colour and more liquid; on further heating for a few minutes the colour disappeared and the consistency became thick and pasty. Water (200 ml.) was added and the mixture warmed until a clear solution was obtained; addition of hydrochloric acid precipitated the product, which was collected and crystallised from 70 % acetic acid. The yield was 14·7 g. and a further 0·75 g. was obtained by dilution of the mother-liquor with water.

For complete purification the acid was dissolved in methylethylketone and the filtered solution evaporated; the residue was then again crystallised from 70 % acetic acid. The compound formed colourless needles having M.P. 207°; it had $[\alpha]_{5461} - 6{\cdot}8°$ ($c = 2{\cdot}72$ in acetic acid; 2 dm. tube). (Found: N, 3·9; I, 38·4. $C_{22}H_{16}O_6N_2I_2$ requires: N, 4·3; I, 38·6 %.)

α-Benzamido-β-[4-(3′ : 5′-diiodo-4′-aminophenoxy)phenyl]propionic acid (II). The nitro-acid (11·7 g.) was dissolved in water (120 ml.) with the aid of sodium hydroxide sufficient just to make the solution alkaline to phenolphthalein. The solution was added to a suspension of ferrous hydroxide prepared by mixing a solution of hydrated ferrous sulphate (30 g.) in water (150 ml.) with one of hydrated barium hydroxide (38 g.) in water (100 ml.); the mixture was heated on the water-bath for 10 minutes. It was then brought to the boil, treated with a slight excess of sodium sulphate and filtered hot; the precipitate was extracted with boiling water and the combined solutions (about 1 litre) acidified with acetic acid and allowed to cool. The first crop amounted to 7·2 g. and a further 2·2 g. was obtained by working up the mother-liquor; the total yield was therefore 84 % of the theoretical. For analysis the acid was recrystallised from 50 % alcohol (charcoal) from which it separated in colourless needles having M.P. 197°. It had $[\alpha]_{5461} - 15{\cdot}0°$ ($c = 2{\cdot}47$ in a mixture of equal parts of N hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 4·4; I, 39·8 %. $C_{22}H_{18}O_4N_2I_2$ requires: N, 4·5; I, 40·4 %.)

α-Benzamido-β-[4-(4'-aminophenoxy)phenyl]propionic acid (III). The preceding compound (9·75 g.) was dissolved in a mixture of *N* sodium hydroxide (280 ml.) and alcohol (200 ml.) and after addition of palladised calcium carbonate (15 g. [*cf.* Harington, 1926]) the solution was shaken in an atmosphere of hydrogen. Uptake of hydrogen ceased at 778 ml. (corrected; about 10 % in excess of the theoretical); the catalyst was filtered off and washed with 50 % alcohol. Alcohol was removed from the combined filtrates by distillation under diminished pressure and the aqueous residue acidified with acetic acid. The product was collected and dissolved in boiling water (200 ml.) with the aid of sodium acetate; the filtered solution was treated at the boiling-point with hydrochloric acid to give maximum precipitation. The yield was 5·1 g. (93 % of the theoretical).

For analysis the acid was recrystallised from 50 % alcohol; it formed colourless needles having M.P. 182·5°. It had $[\alpha]_{5461}-40{\cdot}0°$ ($c=2{\cdot}62$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 7·1 %; $C_{22}H_{20}O_4N_2$ requires: N, 7·4 %.)

d-*Thyronine* (IV). The compound III (1·6 g.) was dissolved in 0·3*N* hydrochloric acid (42·5 ml.) with the aid of heat; on cooling, the solution remained clear. Diazotisation was effected by the gradual addition of powdered sodium nitrite (0·3 g.) the solution being stirred and maintained at 0° throughout and for 15 minutes after the last addition; the diazonium salt crystallised in part. The mixture was diluted with water (40 ml.), and stirring was continued at 0° for 15 minutes longer; the whole was then transferred to a flask and boiled under reflux for 1·5 hours. An equal volume of concentrated hydrochloric acid was added and boiling continued for 6 hours more to effect hydrolysis of the benzoyl group. After cooling, the solution was diluted with an equal volume of water and extracted with ether to remove benzoic acid. The aqueous layer was evaporated to dryness under diminished pressure, and the residue was taken up in water (about 20 ml.); treatment of the hot aqueous solution with sodium acetate caused precipitation of the amino-acid. The yield was about 0·5 g.

The product was purified by dissolution in dilute sulphuric acid and reprecipitation from the hot solution by addition of sodium acetate. It had M.P. 249° (decomp.) and the M.P. was not affected by admixture with *d*-thyronine obtained from *l*-thyroxine (see below). The amino-acid had $[\alpha]_{5461}+13{\cdot}3°$ ($c=0{\cdot}75$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 5·0 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

d-Thyronine from l-*thyroxine.* *l*-Thyroxine (0·075 g.; $[\alpha]_{5461}-3{\cdot}5°$) was dissolved in *N* potassium hydroxide (7·5 ml.) and shaken in an atmosphere of hydrogen after addition of palladised calcium carbonate (0·25 g.); at the conclusion of the uptake of hydrogen the solution was filtered, acidified with acetic acid and evaporated on the water-bath; the recrystallised thyronine amounted to 0·021 g. It had M.P. 247° (decomp.) and $[\alpha]_{5461}+12{\cdot}2°$ ($c=4{\cdot}12$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 0·5 dm. micro-tube). (Found: N, 5·3 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

d-*Thyronine from* l-*3 : 5-diiodothyronine.* For further confirmation a preparation of thyronine was made from *l*-3 : 5-diiodothyronine which is itself known to be stereochemically related to *l*-thyroxine [*cf.* Harington, 1928]. *l*-3 : 5-Diiodothyronine (0·3 g.; $[\alpha]_{5461}-1{\cdot}0°$) was catalytically deiodinated in presence of palladised calcium carbonate (1 g.); the yield of thyronine was 0·128 g. and the product had M.P. 252° (decomp.) and $[\alpha]_{5461}+13{\cdot}9°$ ($c=0{\cdot}753$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 5·1 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

Note on preparation of l-*4-(4′-aminophenoxy)phenylalanine.*

α-Benzamido-β-[4-(4′-aminophenoxy)phenyl]propionic acid (III) (0·5 g.) was boiled under reflux for 4 hours with 20 % hydrochloric acid (10 ml.); the solution was cooled, diluted with water and extracted with ether to remove benzoic acid. The aqueous layer was evaporated to dryness under diminished pressure and the residue taken up in a little hot water. After partial neutralisation with sodium acetate the solution was boiled with charcoal and filtered and the filtrate carefully neutralised to litmus. The product which separated on cooling was collected and recrystallised from boiling water from which it separated in colourless glistening needles. It had M.P. 239° (decomp.) and $[\alpha]_{5461} - 5{\cdot}0°$ ($c = 1{\cdot}2$ in 0·5 *N* hydrochloric acid; 1 dm. tube). The compound was considerably more soluble than thyronine in water; it gave a very intense ninhydrin reaction without previous neutralisation with sodium acetate. (Found: N, 10·3 %. $C_{15}H_{16}O_3N_2$ requires: N, 10·3 %.)

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XIII. THE COMPONENT FATTY ACIDS AND GLYCERIDES OF THE MILK-FAT OF INDIAN CAMELS.

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(*Received December 12th, 1933.*)

The component fatty acids and glycerides of the milk-fat of the camel have been studied in detail, in order to compare them with those of the milk-fats of the cow [Hilditch *et al.*, 1929; 1930; 1931], buffalo [Bhattacharya and Hilditch, 1931], sheep and goat [Dhingra, 1933].

In north-western parts of India milk from camels is utilised as an article of diet by some tribes, but the fat is rarely extracted from the milk and used as a substitute for "ghee" (camel milk-fat is not included in the term "ghee" as defined in the Bombay "Adulteration of Ghee" Act).

The milk-fat used in the present investigation was prepared by churning fresh milk from the animals, and after separating water and caseinogen from the butter, the fat was stored *in vacuo* in sealed amber glass bottles. The milk-fat was obtained in the month of March 1933, when the temperature range was 50°–80° F., from Gujranwala (Panjab) camels, which had been fed partly on green grass but mainly on the tree leaves of *Zizyphus jujuba*, *Acacia speciosa* and *Acacia Arabica*. The yield of the fat from different samples of the milk varied from 2·0 to 2·5 %.

The general characteristics of the milk-fat are given in Table I.

Table I.

Colour	Almost white (cream tinge)
Odour	Buttery, with a slight tallowy odour
Sap. equiv.	259·0
Iodine value	40·8
Acid value	0·2
Reichert-Meissl value	16·4
Polenske value	1·6
Kirschner value	14·3
Setting-point	35·3
Refractive index $n_D^{40°}$	1·4555

The Reichert-Meissl and Kirschner values of camel butter-fat are lower and the saponification equivalent is higher than corresponding characteristics of cow, buffalo, goat and sheep butter-fats, but the Polenske value is considerably lower than that of the goat and sheep butter-fats and more nearly resembles that of cow and buffalo milk-fats.

Component fatty acids of camel milk-fat.

The method followed for the quantitative determination of the component fatty acids was that developed by Hilditch and Jones [1929]. On account of the small amount of the fat available, the mixed fatty acids from 200 g. of fat only

were employed for this purpose. The details of fractionation data are given in Table II, and the final results for the fat are summarised in Table III.

Table II.

(*a*) *Acids volatile in steam.*

No.	g.	B.P. (° C.)	Pressure	Mean equivalent	Acids: Butyric	Caproic	Caprylic	Capric	Oleic	N – S
1	In aqueous solution (4580 ml.)			—	0·90	—	—	—	—	—
2	In recovered ether (1535 ml.)			—	0·05	—	—	—	—	—
3	4·99	35–80	Atmospheric	—	0·13	—	—	—	—	—
4	1·55	80–140	,,	—	0·65	—	—	—	—	—
5	1·02	141–142	,,	—	0·91	—	—	—	—	—
6	1·22	140–141	,,	95·7	0·83	0·39	—	—	—	—
7	0·88	142–160	,,	101·0	0·42	0·46	—	—	—	—
8	0·57	100–110	Reduced	107·7	0·14	0·43	—	—	—	—
9	0·68	112–122	,,	126·4	—	0·40	0·28	—	—	—
10	1·48	Residue	,, (Iodine value 13·0)	183·2	—	—	0·94	0·14	0·21	0·19
					4·03	1·68	1·22	0·14	0·21	0·19

(*b*) *Acids non-volatile in steam.* 190 g.

(Sap. equiv. 261·9; Iod. val. 43·4.)

Lead salt separation.

	g.	%	Corresponding esters: S.E.	I.V.
"Solid" acids S	71·6	48·9	276·1	6·2
"Liquid" acids L	74·8	51·1	274·4	73·9

(*c*) *Fractionation of methyl esters.*

(i) Esters of "Solid" acids S.

Primary fractions: No.	g.	B.P./2 mm.	sap. eq.	I.V.	Refractionations: No.	g.	B.P./2 mm.	sap. eq.	I.V.
					S 11	3·73	120–156	261·0	2·3
					S 12	6·13	155–157	265·2	2·8
S 1	31·96	130–162	270·3	4·0	S 13	4·47	155–157	267·9	3·1
					S 14	4·27	156–158	273·0	4·0
					S 15	5·68	160–168	276·1	5·5
					S 16	2·40	Residue	283·9	8·7
S 2	4·81	160–164	283·4	8·2					
S 3	4·45	165–167	290·2	10·7					
S 4	3·71	Residue	297·8	15·3					

(ii) Esters of "Liquid" acids L.

					L 11	2·17	95–133	214·0	26·8
					L 12	4·10	135–148	250·7	47·4
					L 13	4·70	148–155	267·5	59·7
					L 14	5·12	152–159	270·3	64·4
L 1	38·94	95–180	270·1	70·2	L 15	5·13	158–160	281·9	78·2
					L 16	3·24	158–161	283·3	83·9
					L 17	4·80	158–160	295·8	88·9
					L 18	3·20	Residue	295·1	90·1
L 2	3·85	178–181	296·3	93·5					
L 3	2·00	181–182	295·9	94·2					
L 4	3·28	Residue	308·4	84·6					

The presence of the ordinary $\Delta^{9:10}$-oleic and $\Delta^{9:10,12:13}$-linoleic acids in the unsaturated components of the fat was shown by the identification of a dihydroxy-

Table III. *Summarised experimental data for fatty acids.*

Acid	Volatile acids 3·9 %	Acids non-volatile in steam: Solid acids S 47·0 %	Acids non-volatile in steam: Liquid acids L 49·1 %	Component fatty acids: including unsaponifiable % (wt.)	Component fatty acids: excluding unsaponifiable % (wt.)	Component fatty acids: excluding unsaponifiable % (mols.)
Butyric	2·10	—	—	2·10	2·1	5·9
Caproic	0·88	—	—	0·88	0·9	1·9
Caprylic	0·64	—	—	0·64	0·6	1·1
Capric	0·07	—	1·36	1·43	1·4	2·1
Lauric	—	—	4·56	4·56	4·6	5·7
Myristic	—	3·35	3·93	7·28	7·3	7·9
Palmitic	—	29·22	—	29·22	29·3	28·3
Stearic	—	11·08	—	11·08	11·1	9·7
Oleic	0·11	3·35	35·34	38·80	38·9	34·1
Linoleic	—	—	3·77	3·77	3·8	3·3
Unsaponifiable	0·10	—	0·14	0·24	—	—

stearic acid (M.P. 130°) and a tetrahydroxystearic acid (M.P. 172°) respectively from the products of oxidation of the dilute aqueous solution of the potassium salts of the acids from the penultimate fraction of the "liquid" esters. On hydrolysis of the residual fraction of the "solid" esters, stearic acid was identified.

For comparison, the molar percentages of the component fatty acids of one buffalo, two cow and one sheep milk-fats, along with those of the camel butter, are given in Table IV; while additional information may be gained from consideration of the combined amounts of the four groups of acids given at the foot of the Table.

Table IV. *Comparison of the molar distribution of the component acids of camel, cow, buffalo and sheep milk-fats.*

Fat	Camel butter	Cow butters [Hilditch and Sleightholme, 1931]: Autumn fed English I	Cow butters [Hilditch and Sleightholme, 1931]: Early summer pasture-fed English IV	Sheep butter [Dhingra, 1933]	Buffalo butter No. 2 [Bhattacharya and Hilditch, 1931]
Iodine value	40·8	41·3	41·6	32·1	33·5
Butyric	5·9	8·4	8·9	8·4	11·0
Caproic	1·9	3·5	2·7	5·4	2·8
Caprylic	1·1	2·7	2·0	5·8	1·5
Capric	2·1	2·9	3·0	10·1	2·3
Lauric	5·7	4·1	4·7	6·0	3·3
Myristic	7·9	7·2	10·9	11·8	10·4
Palmitic	28·3	27·1	24·3	20·4	28·7
Stearic	9·7	6·4	5·4	5·4	9·3
Arachidic	—	0·7	—	1·3	0·7
Oleic	34·1	33·9	34·6	22·2	27·8
Linoleic	3·3	3·1	3·5	3·2	2·2
Butyric-lauric	16·7	21·6	21·3	35·7	20·9
Myristic-palmitic	36·2	34·3	35·2	32·2	39·1
Stearic (-arachidic)	9·7	7·1	5·4	6·7	10·0
Oleic-linoleic	37·4	37·0	38·1	25·4	30·0

It is clear that camel milk-fat contains definitely less butyric acid than any of the other milk-fats in this Table. The caprylic and capric acid contents of camel milk-fat resemble those of the milk-fats of the cow and buffalo; sheep and goat

milk-fats, it may be recalled, stand apart in their relatively high proportions of these two acids and in corresponding reductions in their contents of oleic and palmitic acids. The differences between the fatty acids of the camel milk-fat and milk-fats of autumn-fed and early summer pasture-fed English cows is not so marked as between those of the former and the milk-fats of the buffalo, goat or sheep.

The low content of acids volatile in steam in camel milk-fat may partly be due to its xerophytic plant food, or as suggested by Dhingra [1933] in the case of goat and sheep butters, it may be mainly connected with the species of the animal. Possibly the amount of these acids decreases with the size and species of the animals in the decreasing order of sheep, goat, cow, buffalo and camel; but this conclusion requires further confirmation and for this purpose experiments are in progress.

Component glycerides of the camel milk-fat.

The glyceride structure of the fat was studied by separating quantitatively the fully-saturated glycerides present and by determining the component fatty acids in the latter, as described by Hilditch and Jones [1929]; from the combined data for the component fatty acids of the whole fat and of the fully-saturated glycerides, the components of the mixed saturated-unsaturated glycerides present were deduced.

The fat (250 g.) yielded after oxidation crude neutral products (69·9 g.), a portion of which (69·0 g.) was resolved by further purification into: (*a*) 56·2 g., acid value 0·3, (*b*) 4·5 g., acid value 16·1, and (*c*) acidic products, 8·3 g., acid value 90·3. From this, the amount of fully-saturated glycerides in the original fat was determined to be 24·2 % (wt.) or 25·6 % (mols.), corresponding to an "association ratio" of 0·98 mol. of saturated per mol. of unsaturated acids in the mixed saturated-unsaturated glycerides. The component acids of the fully-saturated glycerides are made up as follows:

Table V.

Acid	% (wt.) (Excluding unsaponifiable matter)	% (mol.) (Excluding unsaponifiable matter)
Butyric	3·4	8·9
Caproic	1·0	2·1
Caprylic	0·1	0·1
Capric	2·1	2·8
Lauric	2·2	2·6
Myristic	18·4	18·9
Palmitic	55·0	50·0
Stearic	17·8	14·6

As in cow, goat and sheep butter-fats, the proportion of fully-saturated components of camel milk-fat is a function of the relative amounts of saturated and unsaturated acids in the whole fat. The amount of the fully-saturated glycerides in the present case is somewhat less for a given ratio of saturated to unsaturated acids in the whole fat, than that in cow butters of somewhat similar iodine value. It will be noticed also that the ratio of saturated to unsaturated acids in the mixed saturated-unsaturated glycerides of camel milk-fat is somewhat higher than that of cow butters.

Distribution of the fatty acids in the camel butter glycerides.

The molar percentages of the different groups of fatty acids in the whole fat, and its fully-saturated and non-fully-saturated glyceride components are collected in Table VI. As in Table IV, the data for the milk-fats of two cows, buffalo and sheep are also added for comparison with the camel milk-fat.

Table VI. *Molar distribution of fatty acids in camel, cow, buffalo and sheep milk-fats.*

Section	Acids	Camel butter	Cow butters [Hilditch and Sleightholme, 1931] Autumn fed English I	Cow butters [Hilditch and Sleightholme, 1931] Early summer pasture English IV	[Bhattacharya and Hilditch, 1931] Buffalo ghee, No. 2	[Dhingra, 1933] Sheep butter
Whole fat	Butyric-lauric	16·7	21·6	21·3	20·9	35·7
	Myristic	7·9	7·2	10·9	10·4	11·8
	Palmitic	28·3	27·1	24·3	28·7	20·4
	Stearic (-arachidic)	9·7	7·1	5·4	10·0	6·7
	Oleic-linoleic	37·4	37·0	38·1	30·0	25·4
Fully-saturated	Molar % F.S.G.	25·6	29·1	27·2	34·0	36·8
	Butyric-lauric	16·5	28·6	30·5	25·9	48·3
	Myristic	18·9	13·2	15·1	9·2	15·9
	Palmitic	50·0	43·1	39·5	47·1	26·9
	Stearic (-arachidic)	14·6	15·1	14·9	17·8	8·9
Mixed saturated-unsaturated	Molar % F.S.G.	74·4	70·9	72·8	66·0	67·2
	Butyric-lauric	16·6	19·0	18·3	18·5	28·4
	Myristic	4·2	5·0	9·4	11·1	9·4
	Palmitic	20·8	20·9	18·7	19·2	16·5
	Stearic (-arachidic)	8·0	4·1	2·2	5·8	5·5
	Oleic-linoleic	50·4	51·0	51·4	45·4	40·2

Hilditch and Sleightholme [1931] observed that the component acids of fully-saturated glycerides of six pasture-fed cow butter-fats, which differed widely in iodine value, were almost constant; but the corresponding data for camel milk-fat differ widely from all these, except that the percentage of stearic acid reaches a figure of about 15 % in camel and also in most of the cow milk-fat fully-saturated glycerides. The main differences between the two classes of fully-saturated glycerides are thus the lower percentage of the butyric-lauric acid group and the correspondingly higher proportions of myristic and palmitic acids in the case of the camel butter-fat. The differences between the components of the fully-saturated glycerides of the milk-fat of camels and those of goat, sheep and buffalo butter-fats are much more marked.

The percentages of the components of the mixed saturated-unsaturated glycerides of camel milk-fat are on the whole similar to those of the six pasture-fed cow butters and, to some extent, to those of buffalo ghee; but they differ from those of goat and sheep milk-fats in showing a lower percentage of the butyric-lauric group of acids and a correspondingly higher proportion of unsaturated acids.

SUMMARY.

The fatty acids of the milk-fat of the camel differ from those of cow, buffalo, goat and sheep milk-fats in a lower content of fatty acids volatile in steam. Camel milk-fat can to a great extent be distinguished from the milk-fats of other animals by its lower Kirschner and Reichert-Meissl values. The amount of the lower fatty acids is in decreasing order in the milk-fats of the sheep, goat, cow, buffalo and camel.

The content of the fully-saturated glycerides in camel milk-fat is a function of mean unsaturation, like that of the milk-fats of other animals. The component fatty acids of the fully-saturated glycerides of camel milk-fat are in somewhat different proportions to those in the milk-fats hitherto studied. The composition of the fatty acids of the mixed saturated-unsaturated glycerides of camel milk-fat is little different from that of most cow and buffalo butters, but more so from that of goat and sheep milk-fats.

From the point of view of general properties and of the component glycerides present, there seems no reason why camel milk-fat should not be used as a substitute for cow, buffalo, *etc.*, milk-fats.

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XIV. A MODIFIED SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CAROTENE AND VITAMIN A IN BUTTER.

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(*Received December 19th, 1933.*)

In a previous investigation [Gillam *et al.*, 1933] a spectrophotometric method was developed for the evaluation of carotene, xanthophyll and vitamin A in butter. This involved the separation of xanthophyll from carotene by the well-known partition between methyl alcohol and light petroleum, but although necessary for an original research, it was felt that this method could now be considerably shortened for routine purposes. Experience shows that the carotene : xanthophyll ratio in butter averages 14 : 1 by weight, a fact which, as will be seen later, permits of the approximate estimation of the relatively unimportant xanthophyll without actual determination.

The spectrophotometric evaluation of carotene and xanthophyll depends on the fact that each of these substances exhibits a group of absorption bands in the visible region of the spectrum, the principal one occurring at $463 m\mu$ for carotene and $455 m\mu$ for xanthophyll (in chloroform solution). The amount of either carotenoid present in a solution containing only one of them is proportional to the intensity of its particular absorption band.

The following table shows the average light absorptive power (which is related to the yellow colour) of the carotene and xanthophyll fractions of some 70 samples of English butter, arranged in six sets but actually representing twenty small groups of cattle, of several pure breeds.

Group	No. of samples	$*E^{1\%\text{ dry butter}}_{1\text{ cm.}}$ due to carotene ($463\ m\mu$)	$E^{1\%\text{ dry butter}}_{1\text{ cm.}}$ due to xanthophyll ($455\ m\mu$)	% of total absorption due to carotene ($460\ m\mu$)
A	11	0·00522	0·000348	93·8
B	9	0·00395	0·000285	93·3
C	10	0·00878	0·000468	94·9
D	8	0·00526	0·000333	94·1
E	24	0·00502	0·000371	93·1
F	8	0·01159	0·000391	96·7
	70		Average value	94·0

* $E^{1\%}_{1\text{ cm.}} = \log I_0/I$ for a 1 cm. layer of a solution equivalent to 1 % of dry butter, I_0 = the intensity of the incident light, and I = the intensity of the transmitted light.

Allowing for the slightly different intensities of absorption of carotene and xanthophyll ($E^{1\%}_{1\text{ cm.}}$ $463 m\mu = 1900$ for carotene, and $E^{1\%}_{1\text{ cm.}}$ $455 m\mu = 1700$ for xanthophyll (lutein), when chloroform is the solvent) this absorption ratio of 94 : 6 becomes 14 : 1 for the ratio of carotene to xanthophyll by weight in butter.

These results, and the individual analyses from which they were summarised, show that:

(*a*) On the average, 94 % of the light absorption of butter-unsaponifiable matter in chloroform, at 455–460 $m\mu$, is due to carotene, the remainder being due to xanthophyll.

(*b*) The values for individual samples approximate to this mean value, and as xanthophyll is only a minor constituent of the butter pigment, small departures from constancy do not affect the resulting carotene percentage seriously, if the xanthophyll is estimated at approximately 6 % of the total yellow colour, instead of being actually determined.

The estimation of the carotene value at approximately 94 % of the total yellow colour of the butter, makes it possible to modify considerably the method of evaluation of carotene and vitamin A previously developed, with advantages which more than offset any error likely to occur through departures from constancy in the carotene : xanthophyll ratio.

(*a*) The elimination of the xanthophyll determination removes the need for the partition between light petroleum and methyl alcohol, with consequent saving of much time and material.

(*b*) The spectroscopic work is reduced to half because the unsaponifiable matter is examined as a whole instead of in two separate fractions.

(*c*) The accuracy of the method is increased by the much reduced handling of material well-known to be labile, as well as by the evaluation of the vitamin A in one fraction, instead of adding two results (and therefore two errors) together.

(*d*) The weight of butter required for the determination can be reduced, making a further economy in time and material.

Baumann and Steenbock [1933] have recently reported the results of an examination of fourteen American butters consisting of bulked samples representing the butter obtained in their particular district in each month of the year. They used a spectrophotometric method for both the carotene and vitamin A determinations but did not consider it necessary to correct their carotene values for xanthophyll. They pointed out, however, that in the case of the unsaponifiable matter of one particular August butter, approximately 95 % of the pigment was not extractable from light petroleum by methyl alcohol, and moreover, its absorption spectrum was characteristic of carotene. Thus the value they found for the carotene : xanthophyll ratio in this particular butter is very close to the value here shown to be the average of a large number of butters.

The modified procedure.

Preparation of the unsaponifiable matter. A weighed portion of the butter (100 g. of winter butter, or 25–50 g. of summer butter or half quantities if the antimony trichloride test is not being used) of known moisture content, is saponified for half an hour with a slight excess of colourless 20 % alcoholic potash, and the soaps are poured into about three volumes of water[1]. The solution is extracted with ether until no more colour is removed, the extracts being bulked and washed carefully with several portions of water. The whole of the washed ether extract is then freed from solvent by evaporation in nitrogen. If the spectroscopic examination is not to be carried out at once this evaporation should be completed in a brown glass ampoule, the residue being finally sealed off in

[1] The colourless alcoholic potash recommended is obtained by using alcohol which has been previously boiled over, and distilled from, either metallic sodium or caustic soda.

nitrogen. Otherwise, the residue is dissolved in chloroform, made up to 10–20 ml. in a graduated flask and examined immediately.

Determination of the carotene. The solution now represents a known weight of butter per 100 ml. The intensity of absorption at 455–460 $m\mu$ is measured either photographically, using an apparatus such as the Hilger E3 quartz spectrograph with a suitable photometer, or visually, using a spectrophotometer of the type of the Hilger-Nutting instrument[1].

We have found the visual method to be the better of these two, as there is less danger of destruction of the carotenoids by the ultra-violet rays of the light source (where an arc or spark is used with quartz apparatus) in the photographic method.

The value so obtained is expressed as $E^{1\%\text{ butter}}_{1\text{ cm.}}$ at 455–460 $m\mu$ and the absorption due to carotene is taken as 94 % of this value. To calculate the percentage of carotene in the butter, use is made of the fact that $E^{1\%}_{1\text{ cm.}}$ 463 $m\mu$ ($CHCl_3$) = 1900 for pure carotene [Gillam *et al.*, 1932]. The result is conveniently expressed as mg. carotene per 100 g. dry butter.

Determination of the vitamin A.

(*a*) *By means of the ultra-violet absorption band.* It is now generally accepted that the absorption band at 328 $m\mu$ shown by certain fish-liver oils [Morton and Heilbron, 1928] is a definite property of vitamin A. This is also exhibited by butter [Morton and Heilbron, 1930] and provides a means of evaluation of the vitamin A content. In the case of butter-unsaponifiable matter the absorption at 328 $m\mu$ is a summation of that due to vitamin A together with the smaller contributions due to carotene and xanthophyll. We have found that the absorption due to carotene *plus* xanthophyll, at 328 $m\mu$, is given by dividing the observed value of $E^{1\%}_{1\text{ cm.}}$ for these substances at 455–460 $m\mu$ by the factor 6·5. This correction is usually of the order 10–25 % of the gross absorption at 328 $m\mu$. From the value of $E^{1\%\text{ butter}}_{1\text{ cm.}}$ at 328 $m\mu$, due to vitamin A, it is possible to determine the vitamin A content in mg. per 100 g. dry butter by making use of the value $E^{1\%}_{1\text{ cm.}} = 1600$ given by Carr and Jewell [1933] for their richest concentrate. This value may need correction if vitamin A is concentrated further (*cf.* Karrer and Morf [1933], who have prepared a concentrate of $E^{1\%}_{1\text{ cm.}}$ (328 $m\mu$) = 1700).

(*b*) *By the spectroscopic blue test with antimony trichloride.* If the solution of butter-unsaponifiable matter is treated with antimony chloride in chloroform the blue colour formed is largely due to vitamin A, but some small part is also due to carotene and, to a lesser extent, to xanthophyll. If, however, the intensity of absorption of the blue solution is determined at 585 $m\mu$ with a visual spectrophotometer, according to the technique already described for liver oils [Heilbron *et al.*, 1931] and applied to butters [Gillam *et al.*, 1933], the value so obtained can be regarded as being due to vitamin A and carotene only, the xanthophyll value here being negligible. As therefore the absolute carotene value is known from the direct absorption and as the blue value of pure carotene is also known ($E^{1\%}_{1\text{ cm.}}$ 585 $m\mu$ = 420), it is possible to calculate the contribution due to carotene. The difference gives the blue value due to vitamin A in terms of $E^{1\%\text{ butter}}_{1\text{ cm.}}$ at 585 $m\mu$, and as the purest distillates have a value $E^{1\%}_{1\text{ cm.}}$ 585 $m\mu$ = 2600 [Heilbron *et al.*, 1932] in chloroformic antimony trichloride, the vitamin A content can be

[1] Although pure carotene has its principal absorption band at 463 $m\mu$ for chloroform solution, in the presence of butter-unsaponifiable matter this lies nearer to 455 $m\mu$. The intensity of absorption however is not perceptibly altered by taking the measurement anywhere between 455 and 460 $m\mu$.

calculated in mg. per 100 g. butter. The results obtained in this way are always low, apparently owing to the presence in the original butter of substances which act as inhibitors of the blue colour. This is in agreement with the findings of Booth *et al.* [1933] who have shown that the blue values of butter (in Lovibond units) exhibit considerable inhibition and that the inhibition shows a seasonal variation.

In view of these facts the spectroscopic modification of the antimony trichloride blue test is not recommended for the general determination of the vitamin A content of butter, but it is included here as a secondary test if for any reason, such as the presence of unsaponifiable artificial colouring matter, the ultra-violet absorption spectrum method is interfered with.

An analysis of the carotene, xanthophyll and vitamin A values of some 70 English butters consisting largely of winter samples yields the following data (obtained by the full method):

	mg. per 100 g. dry butter			Estimated growth-promoting effect* in International units per g.
	Carotene (1900 basis)	Xanthophyll (1700 basis)	Vitamin A (1600 basis)	
Mean values (70 samples)	0·327	0·024	0·473	11·1
Maximum	0·88	0·075	0·922	24·2
Minimum	0·025	0·003	0·158	2·9
Mean value cows at grass (12 samples)	0·60	0·029	0·72	18·0
Mean value stall-fed cattle (58 samples)	0·272	0·0227	0·420	9·7

* Calculated from the numerical values on the assumption that vitamin A is 1·66 times as potent as carotene [Carr and Jewell, 1933]. This value may need revision when really representative values are available.

In connection with the application of this method the following points should be noted.

(*a*) All values recorded were obtained on unsalted butters and are expressed on dry butter-fat. To obtain comparable results on salted butter the water and salt should be estimated and the carotenoid values calculated on the butter-fat.

(*b*) The majority of these butters were made from the milk of pure-bred Shorthorn cows, with a sufficient number of samples from pure-bred Ayrshire and South Devon herds to show that the carotene : xanthophyll ratio applies approximately to these also. A few unclassified samples have also been tested with good results.

(*c*) When butters contain added colouring matter, any method for carotenoid determination depending on yellow colour will give faulty results, hence the treatment will need modification according to the nature of the colouring matter. If this should interfere with the absorption spectrum determination of vitamin A, the antimony chloride test can be called into service.

Summary.

1. Values for the carotene, xanthophyll and vitamin A contents of seventy English butters have been obtained spectrophotometrically. Mean values are recorded.

2. It is shown that, on the average, the ratio of carotene to xanthophyll in butter is 14 : 1 by weight.

3. It is further pointed out that if 94 % of the light absorption of butter-unsaponifiable matter at 455–460 $m\mu$ is taken as being due to carotene, the results on individual samples are sufficiently close to those actually determined after removal of the xanthophyll to justify the approximation by the economy effected in time and material.

4. The spectrophotometric method previously developed for the determination of carotene and vitamin A in butter has been improved for routine purposes. Full details of the modified method are given.

My thanks are due to Prof. I. M. Heilbron for his interest and support, to Dr R. A. Morton for the use of his laboratory and to the Staff of Messrs Imperial Chemical Industries, Ltd., Agricultural Research Station, Jealott's Hill, for their invaluable collaboration.

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XV. THE NUCLEOTIDE COMPONENT OF MAMMALIAN CARDIAC MUSCLE.

I. THE COMPOSITION AND CHEMICAL PROPERTIES OF THE NUCLEOTIDE.

By FLORENCE BEATTIE, THOMAS HUGH MILROY and ROBERT WILLIAM MAGILL STRAIN.

II. THE BIOLOGICAL AND CO-FERMENT PROPERTIES OF THE CARDIAC DINUCLEOTIDE.

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(*Received December 23rd, 1933.*)

I. THE COMPOSITION AND CHEMICAL PROPERTIES OF THE NUCLEOTIDE.

The presence of an adenosinetriphosphoric acid in mammalian skeletal muscle was discovered independently by Fiske and Subbarow [1929] and Lohmann [1929]. Barrenscheen and Filz [1932] have recently suggested that the labile phosphoryl linkages in this nucleotide are at least in part with the NH_2 of the adenine, their opinion being based mainly upon the difficulties associated with the deamination without dephosphorylation (of the loosely bound phosphorus), whether this is brought about by chemical or by enzymic processes. From an examination of their acid hydrolysis experiments it is evident that the nucleotide which they had prepared from skeletal muscle behaved differently from pure adenosinetriphosphoric acid for, even after 1–3 hours' treatment with *N* HCl at 100°, only 80–85 % of the loosely bound phosphoric acid was set free, while the same compound prepared by others readily gives off completely two of the three phosphoric acid molecules within ten minutes under the above conditions. From a study of the acid hydrolysis curves of nucleotides certain important differences in the nature of the linkages in purine nucleotides of different origin may be readily observed. Even in the cases of the adenosinemonophosphoric acids of yeast and muscle, it is evident that there is a characteristic difference in the firmness of the single phosphoryl linkage with an alcohol group of the pentose. Thus the phosphoryl linkage of the yeast derivative can be readily broken by acid hydrolysis, while those of the muscle compound and its deaminated derivative, inosinic acid, offer a much greater resistance [Levene and Jorpes, 1929]. The position of the phosphoric acid linkage in the muscle derivatives has been definitely established, namely to the terminal alcohol group of the ribose (C_5), while that of the similar linkage in the adenylic acid of yeast is probably the same as in guanylic acid [Levene and Harris, 1932] namely to C_3 of the ribose. The lability of the phosphoryl linkage in the yeast derivative is of much the same order as that of the two loosely bound

phosphoryl groups in adenosinetriphosphoric acid (a.t.p.) of skeletal muscle. The ease with which the labile linkages in the latter may be broken is shown in Embden's method of preparing adenylic acid from muscle, when under the action of $Ca(OH)_2$ at room temperature the original molecule loses two of the three phosphoric acid molecules. As will be seen later the nucleotide of cardiac muscle shows certain characteristic differences from that of skeletal muscle, due to the number and possibly also the position of the phosphoryl linkages. It is necessary always to bear in mind that the methods employed in separating the salts of these nucleotides may give rise to alterations in the character of the linkages. Fischer [1914] in his investigation of synthetic theophylline glucoside-phosphoric acid referred to the ease with which the phosphoryl linkage might be altered with reaction, thus the original monobasic acid with two of the OH groups of the phosphoric acid fixed to alcohol groups of the glucose might readily be changed to a dibasic acid with one only of the OH groups fixed to a single alcohol group.

In this communication special attention will be directed to the adenyl nucleotide in cardiac muscle, but it will be necessary to compare it with the closely allied substance met with in skeletal muscle. There are only two definite references, both very brief, to the adenyl derivative of heart muscle. One of these is by Lohmann [1931] in which the following characteristics of this body are just mentioned. The barium salt on neutral hydrolysis was found to furnish adenylic acid and pyrophosphoric acid, and the ratio of the loosely bound to the firmly bound phosphorus was found to be 1 : 1, instead of 2 : 1. The second reference occurs in a paper by Deuticke [1932] dealing with the action of the heart nucleotide on the uterus of the guinea-pig, in which he briefly mentions a statement made by Embden at a congress in Wiesbaden in 1932 to the effect that the substance is probably a dinucleotide consisting of one molecule of adenosinetriphosphoric acid and one of adenosinediphosphoric acid in which the ratio of N : P is therefore 10 : 5. Ostern and Mann [1933], dealing with the deamination process in heart-muscle, refer briefly to the statements made by Lohmann and Embden, but they did not themselves analyse the compound.

Evidence may be obtained very readily of the presence in heart-muscle of a substance possessing approximately two labile H_3PO_4 molecules to one firmly bound. Thus in the trichloroacetic acid extract of the muscle without separation of the substance, as also in the calcium hydroxide precipitate and in the mercuric acetate precipitate, the existence of such a substance is easily shown. When, however, further purification of the nucleotide is carried out in the way to be described, certain differences between the skeletal and cardiac derivatives become evident. The method of separation of the silver salt follows, generally speaking, the procedure originally adopted by Fiske and Subbarow for the skeletal muscle derivative, but their description is very brief.

Preparation of the silver salt of the cardiac nucleotide.

The same salt may be obtained from the hearts of the rabbit, cat, dog and ox, but in order to obtain larger quantities it is advisable to use the heart of the horse. The heart was removed immediately after death, the cavities being slit open and washed out with normal saline solution. The whole organ while still showing fibrillary contractions was placed in a large basin surrounded by freezing mixture and immediately taken to the laboratory for extraction. The more muscular parts were removed and minced. In this way about one kg. of muscle could be obtained, which was then extracted with two volumes of 5 % trichloroacetic acid, filtered, neutralised and again filtered. About two litres of

protein-free extract were obtained. Acetic acid was added up to 2 % and then saturated mercuric acetate solution in 2 % acetic acid until precipitation was complete. The deposit was separated by centrifuge and after washing with weak mercuric acetate solution the mercury was removed by H_2S and the latter by aeration. This solution was found to contain per g. muscle; total P, 0·029 mg.; P as orthophosphoric acid, none; P as orthophosphoric acid after hydrolysis for 10 mins. with *N* HCl at 100°, 0·02 mg. The solution was then concentrated *in vacuo* at 40° to a volume of approximately 150 ml. To this 20–30 ml. 20 % $CaCl_2$ and ethyl alcohol slightly acidified with HCl were added until precipitation was complete. The precipitate, after repeated solution and reprecipitation, was finally washed free from chlorine by 50 %, 70 % and finally absolute alcohol. After drying, the salt was dissolved in weak nitric acid and 10 % $AgNO_3$ added until the separation of the crystalline salt was complete. The precipitate was thoroughly washed with weaker alcohols and then with ethyl alcohol and ether and finally dried *in vacuo* at 80° over P_2O_5.

The yields from heart muscle were always much less than from skeletal muscle. The silver salt of the nucleotide in skeletal muscle was prepared in the same way.

Attention was directed especially to the estimation of the total nitrogen and phosphorus, the ribose and the silver.

Methods of analysis.

Nitrogen; by micro-Kjeldahl.

Phosphorus; by Fiske and Subbarow's method.

Ribose; the method employed was to determine the reducing value by Hagedorn and Jensen's method after half an hour's hydrolysis at 100° with *N* HCl to break the glucosidic linkage. Levene in his determinations of the pentose in the purine nucleotides found that the maximum reducing power was reached after about half an hour's acid hydrolysis and that the yield reached about 96 % of the theoretical value. He determined the reducing value of pure *d*-ribose and found it to be (by Hagedorn and Jensen's method) 87·5 % of the glucose value. Our calculations of the ribose value are based upon these data given by Levene. The furfuraldehyde yield was also determined and was always found to be higher using the method of Andrews and Milroy [1933] than with the Hoffman method.

Silver was estimated by the usual thiocyanate method. In all cases the silver salts analysed were free from inorganic phosphate. As will be seen the analyses of the silver salt of the skeletal muscle derivative correspond closely to a salt with the formula $C_{10}H_{13}O_{13}N_5P_3Ag_3$.

From Lohmann's electrotitration curves [1932] the acid appears to possess in its unbroken form four acid valencies, three primary and one secondary. In the silver salt two of the three silver atoms seem to be taken up by the primary acid groups in the labile diphosphoryl linkage and the other by the primary acid OH of the stable phosphoric acid linked to the terminal alcohol group in the ribose. If the acid be regarded, as appears probable, as tetrabasic with a labile anhydro-diphosphoryl linkage, the above formula includes one molecule of water.

	Calculated for $C_{10}H_{13}O_{13}N_5P_3Ag_3$ (827·84) %	Found Spec. 1 %	Found Spec. 2 %
N	8·46	8·62	8·29
P	11·24	11·18	11·08
Ribose	18·12	18·00	17·78
Silver	39·09	39·12	38·85

The above are the analyses of the silver salts of two specimens of the skeletal derivative obtained from different sources.

The salt therefore corresponds to one with N : P = 5 : 3 and the agreement with the calculated values for silver and ribose is fairly close.

When the analyses of the cardiac derivative are examined such an agreement with an adenosinetriphosphate is no longer evident. For comparison with other possible nucleotide forms, the values found are to be compared not only with the silver salt of adenosinetriphosphate (I), but also with the theoretical values for a corresponding adenosinediphosphate (II) and for a dinucleotide consisting of an equimolecular mixture of these two (III).

	Calculated for			Found	
	I	II	III		
	$C_{10}H_{13}O_{19}N_5P_3Ag_3$ (827·84) %	$C_{10}H_{13}O_{10}N_5P_2Ag_2$ (640·94) %	$C_{20}H_{26}O_{29}N_{10}P_5Ag_5$ (1468·78) %	Spec. 1 %	Spec. 2 %
N	8·46	10·92	9·53	9·60	9·51
P	11·24	9·67	10·56	9·92	10·00
Ribose	18·12	23·40	20·43	19·80	19·50
Silver	39·09	33·66	36·72	34·82	35·40

Specimens I and II have N : P = 10 : 4·7 in place of 10 : 5 and the silver values are below the theoretical for such a dinucleotide. The salts are however much more closely allied to a mixture of one molecule of adenosinetriphosphate with one of the diphosphate than either of these in the pure form. That the labile phosphoryl linkages in the cardiac muscle derivative differ slightly from those in that of the skeletal muscle is evident from the results of hydrolysis with *N* HCl. Moreover one of the three labile phosphoric acid molecules may be set free on precipitation of the dinucleotide as a lead salt, giving then an adenosinediphosphate with a ratio of labile to stable P of 1 such as Lohmann observed. An example of this change having taken place may be given. A portion of the calcium salt of the cardiac derivative (0·2 g.) after removal of calcium was precipitated with normal lead acetate, the lead salt decomposed in the usual way and the solution made up to the same volume as before precipitation. The phosphorus distribution before and after precipitation by lead was as follows (mg. P per ml.).

	Before	After		
Orthophosphoric acid	nil	0·064	orthophosphoric	0·064
After 10 mins. acid hydrolysis	0·177	0·177	labile	0·113
Total	0·290	0·290	stable	0·113

In the solution prior to precipitation with lead 61 % of the total P was set free on acid hydrolysis (*N* HCl), corresponding therefore to 3 labile to 2 stable groups as in an equimolecular mixture of the tri- and di-phosphates. If the solution had contained the triphosphate alone, 66 % of the total P would have been in labile form.

Acid hydrolysis of the cardiac nucleotide.

In all such hydrolysis experiments with *N* HCl special attention requires to be directed to the amounts of inorganic phosphate set free within the first 10–30 mins., because, subsequent to the setting free of the labile P, the rate of hydrolysis of the stable P is very slow and for all such adenyl nucleotides as occur in muscle fairly constant.

The procedure adopted was as follows. One ml. of the solution containing a known quantity of the cardiac (or skeletal) derivative was mixed with 1 ml. of 2*N* HCl or 2*N* acetic acid and kept at 100° in sealed tubes for the periods stated.

After rapid cooling the solutions were neutralised and made up to a volume corresponding to the standard solution concentration (0·4 mg. P per 100 ml.), so that the colorimetric determinations might be carried out under strictly comparable conditions.

Table I. *Hydrolysis in* N *HCl.*

Skeletal muscle adenosinetriphosphoric acid.

Time (mins.)	(1) P as orthophosphate mg. per ml.	(2) % of total P	(3) % of labile P (2 in 3)
0	0		
10	0·137	67	100
15	0·137	67	
30	0·140	68	
60	0·140	71	
Total P	0·204		

Cardiac muscle dinucleotide.

			% of labile P (3 in 5)
0	0		
10	0·123	60	100
15	0·131	64·5	
30	0·137	67·4	
60	0·141	69·4	

Table II. *Hydrolysis in* N *acetic acid.*

Skeletal muscle adenosinetriphosphoric acid.

Time (mins.)	(1) P as orthophosphate mg. per ml.	(2) % of total P	(3) % of labile P
0	0		
10	0·020	9·8	14·7
15	0·030	14·7	22·0
30	0·046	22·5	33·8
60	0·073	35·2	53·6
120	0·125	61·2	91·8
210	0·136	67·0	100·0
Total P	0·204		Labile P 0·136

Cardiac dinucleotide.

0	0		
10	0·036	8·5	14·2
15	0·046	11·1	18·2
30	0·103	24·5	40·8
60	0·143	34·0	56·7
120	0·195	46·4	77·3
210	0·252	61·4	100·0
Total P	·420		Labile P 0·252

The skeletal nucleotide loses 2/3 of its P after 10 mins. hydrolysis with *N* HCl, while from the cardiac one under the same conditions 3/5 of the total P is set free. On hydrolysis with *N* acetic acid the same changes take place after boiling for

210 mins. After 120 mins. the proportion of the loosely bound P split off from the cardiac derivative is distinctly less than from the skeletal one.

Alkaline hydrolysis of the cardiac dinucleotide.

Lohmann and others have frequently referred to the liberation of orthophosphate or pyrophosphate from the breakdown of adenosinetriphosphoric acid by $Ca(OH)_2$ and $Ba(OH)_2$. It is often impossible to be certain from statements in the literature whether the precipitates of so-called calcium or barium pyrophosphates were purely inorganic or not.

Precipitates obtained after half an hour's hydrolysis in $Ca(OH)_2$ always contain a fairly high percentage of N, although the main product, adenylic acid, forms a soluble calcium salt.

Mixtures of one volume of the solutions of the two nucleotides with one volume of saturated $Ca(OH)_2$ were kept at 100° in sealed tubes for the periods stated, and the orthophosphate at each stage was determined. The mixtures were shaken throughout.

The percentage of labile P in the adenosinetriphosphoric acid is calculated as 66 % of the total, that of the dinucleotide as 60 %.

Table III.

	Skeletal adenosinetriphosphoric acid			Cardiac dinucleotide		
	1	2	3	1	2	3
Time (mins.)	P as orthophosphate mg. per ml.	% of total P	% of labile P	P as orthophosphate mg. per ml.	% of total P	% of labile P
0	0					
5	0·029	30·2	45·3	0·043	43·0	71·6
10	0·047	48·9	73·4	0·045	45·0	75·0
15	0·047	48·9	73·4	0·047	47·0	78·3
30	0·049	51·0	76·5	0·047	47·0	78·3
90	0·066	68·7	100·0	0·050	50·0	83·3
120	0·066	68·7	100·0	0·064	64·0	100·0
180	—	—	—	0·064	64·0	—
	Total P	0·096			Total P	0·100

Difficulties arise in the determination of the rates of hydrolysis with $Ca(OH)_2$ owing mainly to the fact that the salts to be acted upon are not in a state of solution but in one of fine suspension. After the loosely bound phosphoric acid molecules have been set free, the calcium salt of adenylic acid along with varying amounts of adenosine should pass into solution. As it is the amount of ortho-

Table IV.

Time (mins.)	P as orthophosphate mg. per ml.	%
0	0	
5	0·077	48·1
10	0·102	63·6
15	0·130	81·2
30	0·130	81·2
90	0·160	100·0
120	0·160	
	Total P 0·160	

phosphate which is being estimated at the different stages, the rate of hydrolysis of the inorganic pyrophosphate by $Ca(OH)_2$ requires to be known as the loosely bound phosphorus in the nucleotides may be set free in this form. Table IV gives the results of the hydrolysis of sodium pyrophosphate with $Ca(OH)_2$.

When the results of the hydrolysis of the three compounds are examined, it is evident that one factor which may determine the rate of orthophosphate formation from the organic substances is the rate of change of pyrophosphate into orthophosphate. That this is not the only factor in the case of the dinucleotide is evident from the greater initial rate at which orthophosphate is set free from it compared with inorganic pyrophosphate. The labile portion of adenosinetriphosphoric acid behaves somewhat similarly to the inorganic salt on alkaline hydrolysis, just as it does in the case of hydrolysis with *N* HCl. At present no definite evidence is obtainable regarding the position of the labile phosphoric acid molecules in these nucleotides, but it appears probable that there is an anhydrodiphosphoryl linkage in the triphosphoric acid which may combine in the case of dinucleotides with a secondary alcohol group of the ribose in either an adenosinemonophosphate or diphosphate.

II. THE BIOLOGICAL AND CO-FERMENT PROPERTIES OF THE CARDIAC DINUCLEOTIDE.

THE definite biological actions of the adenosinetriphosphoric acid of skeletal muscle origin and the function of that nucleotide in lactic acid production suggest that an investigation into the pharmacology and co-ferment properties of the cardiac dinucleotide would be of special interest for several reasons. In the first place the substance is a natural component of the heart-muscle, and as such its actions on the heart and vessels are of importance, and in the second place, as chemically it presents certain characteristic differences from the derivative of skeletal muscle, any difference in action between the two might be explicable from the standpoint of chemical constitution. Finally, as proprietary preparations of tissue nucleotides and nucleosides are in routine use in the treatment of cardio-vascular disease, any further investigation into the pharmacology of such substances is of value.

Drury and Szent-Györgyi [1929] have studied the biological actions of a cardiac derivative which, as they deduced from careful analyses, was undoubtedly adenylic acid. They refer only briefly to the action of Fiske's adenosinetriphosphate (probably from skeletal muscle).

In all the following experiments the calcium salt of the cardiac dinucleotide was used.

The results are exactly comparable with those of Gillespie [1933] for the skeletal muscle nucleotide, in most cases both salts being tried on the same preparation.

Biological actions.

1. *The heart.* (*a*) Doses of 1 in 50,000 of the cardiac nucleotide perfused through the isolated frog's heart cause no alteration in the normal beat. Doses of 1 in 25,000 produce an A.V. block which passes off on perfusion with fresh Ringer's solution.

(*b*) In the isolated perfused rabbit's heart, doses of 0·1 to 0·2 mg. of the heart dinucleotide added to the perfusion fluid cause first a diminution in rate and amplitude of the beat followed in a few seconds by augmentation of the beat and an increased coronary outflow.

2. *Blood pressure.* Doses as small as 0·01 mg. lower the carotid arterial pressure of the rabbit. The fall in pressure is not so sudden as that produced by vagal stimulation. The fall is preceded by a short rise. The whole effect is quite transitory, and complete recovery takes place in a few seconds. This was also seen after atropine.

3. *Perfusion of vessels.* Frogs' vessels were perfused in the ordinary way. Doses of 0·2 mg. injected at the point of entrance of the perfusion fluid doubled the drop rate in about $1\frac{1}{2}$ mins.

4. *Uterus.* Concentrations of 1 in 250,000 of the cardiac dinucleotide increase the tone of the virgin guinea-pig's uterus perfused by the Sawasaki technique and finally give rise to spontaneous contractions. This is the same effect as is described by Deuticke [1932] in connection with Embden's heart nucleotide.

5. *Intestine.* Concentrations of 1 in 50,000 and less lower the tone of the perfused small intestine of the rabbit. This is a different effect from that produced by the voluntary muscle nucleotide, which causes a marked increase in tone immediately after the fall [Gillespie, 1933]. Using the intestine as a test object it was shown that dialysates of saline extracts of heart muscle owe their biological action to the dinucleotide component.

The action of the cardiac dinucleotide on the amphibian and mammalian heart and vessels and on the perfused uterus closely resembles, both qualitatively and quantitatively, the action of the voluntary muscle nucleotide as described by Gillespie. Voluntary muscle nucleotide produces a fall in intestinal tone followed by a marked rise. Only the fall has been observed with the cardiac derivative.

Ferment actions of the cardiac nucleotide.

The glycolytic ferment of voluntary muscle consists of two fractions—the colloidal ferment and a co-ferment which Meyerhof and Lohmann [1928] have identified with adenylpyrophosphate. Little is known of the glycolytic ferment system in the heart, and as the dinucleotide just described has been tested as co-ferment on cardiac extracts acting on artificial substrates of starch or glucose with orthophosphate, the results of this investigation help to complete the comparison of the two types of nucleotide. Lambs' hearts fresh from the abattoir were used for these experiments.

The first experiments show that an active ferment system can be extracted from the mammalian heart muscle with ice-cold saline. This activity is shown by the appearance of lactic acid in the incubation mixture containing starch and inorganic phosphate and by the disappearance of some of the orthophosphate due to the synthesis of esters. For example:

2nd and 3rd saline extracts incubated 2 hrs. at 25° with starch substrate.

m.mols ester produced	...	...	...	5·6
m.mols lactic acid produced	...	...	...	5·0

When the extract has been kept for 24 hrs. at 4° it no longer produces these effects. The addition of a small amount of the cardiac dinucleotide, or the

adenosinetriphosphoric acid of skeletal muscle, restores the activity of the extract. The following experiment will illustrate the type of action.

2nd and 3rd saline extracts, kept 24 hrs. at 4°, incubated 3½ hrs. at 25° with starch substrate.

1. Extract + substrate + no addition.
   m.mols ester produced ... ... 0·39
   m.mols lactic acid produced ... ... nil
2. Extract + substrate + 0·1 mg. skeletal adenosinetriphosphoric acid per ml.
   m.mols ester produced ... ... 0·70
   m.mols lactic acid produced ... ... 0·79
3. Extract + substrate + 0·1 mg. cardiac dinucleotide per ml.
   m.mols ester produced ... ... 2·10
   m.mols lactic acid produced ... ... 2·00

The cardiac dinucleotide reactivates the extract to a greater degree than does the nucleotide of voluntary muscle. It was found that this occurred also in the case of the reactivation of voluntary muscle extracts. Haarman [1932], in a paper published while these experiments were being carried out, points out that the preferential substrate for heart glycolysis is glucose. Again using the extract described above, but with glucose in place of starch as substrate, the results obtained were as follows:

1. Extract + substrate (glucose) + no addition.
   m.mols ester produced ... ... 0·6
   m.mols lactic acid produced ... ... 0·1
2. Extract + substrate (glucose) + 0·1 mg. skeletal muscle adenosinetriphosphoric acid per ml.
   m.mols ester produced ... ... 0·7
   m.mols lactic acid produced ... ... 1·0
3. Extract + substrate (glucose) + 0·1 mg. cardiac dinucleotide per ml.
   m.mols ester produced ... ... 0·0
   m.mols lactic acid produced ... ... 2·0

In these and other examples it was seen that the extract did not show a preference for glucose, though the observation was confirmed that the heart could use this substrate. It is evident, however, that while esterification is a feature of lactic acid production from starch, this is not the case when the substrate is glucose. Traces of ester may be found with glucose as substrate due to the glycogen content of the original extract. When minced heart muscle is added to an artificial substrate, it is found that, while starch can be used, there is a definite preference for glucose. As in the case of the saline extracts there is no tendency for esters to accumulate when the glucose substrate is used:

3 g. specimens of minced muscle were allowed to act on the phosphate, glucose and starch substrates in a total volume of 20 ml. for 2½ hrs. at 25°.

1. Glucose substrate.
   Orthophosphate used up ... ... 0·52 mg. P in 20 ml.
   Lactic acid produced ... ... 1·92 mg. in 20 ml.
2. Starch substrate.
   Orthophosphate used up ... ... 2·14 mg. P in 20 ml.
   Lactic acid produced ... ... 0·60 mg. in 20 ml.

It also has been found that a more powerful ferment system can be extracted from muscle which has been autolysed for 2 hours than from fresh muscle. The extract of autolysed muscle can be made still more active by addition of co-ferment. It is evident that during autolysis the colloidal component of the ferment system undergoes some change which facilitates its extraction.

The ferment system of cardiac muscle is, on the whole, not nearly so active as the corresponding system in voluntary muscle.

That glucose should be available as an immediate source of energy for the heart, whether or not glycogen is present, is of great interest. The glycolytic ferment system of voluntary muscle is incapable of using glucose directly. This means that, apart from the smooth muscle tissues with their slow rate of metabolism, the heart has an option on sugars in the circulation, a mechanism which must be of considerable importance in maintaining the heart beat in cachexia and privation when glycogen is low. It may also explain in part the therapeutic value of parenteral glucose saline in the treatment of shock and allied conditions.

Summary.

1. The nucleotide of cardiac muscle differs chemically from the adenosinetriphosphoric acid of voluntary muscle, being more closely allied to a dinucleotide composed of one molecule of adenosinediphosphoric acid and one molecule of adenosinetriphosphoric acid.

2. This is shown by (*a*) its chemical composition and (*b*) its behaviour on hydrolysis at 100° with *N* HCl. Approximately 60 % of its total P is in the labile form in place of 66–67 % in adenosinetriphosphoric acid.

3. The pharmacological actions are very similar to those of the voluntary muscle derivative, the only distinctive difference being observed in the reaction of the intestinal musculature, the dinucleotide lowering the tone without a subsequent rise, while adenosinetriphosphoric acid has the double action.

4. The heart dinucleotide has a more powerful co-ferment action as regards the reactivation of inactive ferment extracts both of voluntary and cardiac muscle.

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XVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVI. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH.

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(*Received December 29th, 1933.*)

During an examination of a sample of mouldy Italian maize our late colleague, Mr J. H. V. Charles, isolated a new species of *Penicillium* belonging to Thom's group "The Monoverticillata-Ramigena" [Thom, 1930]. A description of this mould, which has been named *P. Charlesii*, has been published elsewhere [Smith, 1933]. When grown on Czapek-Dox solution or on the medium which we have called Raulin-Thom solution [Clutterbuck *et al.* 1932] the culture medium becomes very strongly acid, reduces $KMnO_4$ instantly in the cold, gives an intense orange or scarlet colour with ferric chloride, and on addition of excess of alcohol gives a voluminous sticky precipitate. In addition, if the period of incubation is sufficiently prolonged, the metabolism solution becomes definitely laevorotatory.

Addition of acetone to the evaporated metabolism solution precipitated a mixture of two new polysaccharides, one of which was a polygalactose and the other a polymannose. The syrupy precipitate obtained with acetone both from Czapek-Dox solution and Raulin-Thom solution gave, on trituration with absolute alcohol and ether, an amorphous hygroscopic powder with a high content of ash consisting chiefly of phosphates. Most of this ash, however, could be removed by precipitation with alcohol.

The aqueous solution of the crude product from Raulin-Thom medium was acid to litmus, with an average rotation of $[\alpha]_{5790} = -40°$, and had no action on Fehling's solution or on iodine. Its behaviour on hydrolysis suggested the presence of two polysaccharides. Treated at 100° with $0{\cdot}4N$ sulphuric acid the rotation changed from $-40°$ to $+61°$, but on pouring the equilibrium solution into alcohol a considerable portion of a polysaccharide was precipitated. With $3N$ sulphuric acid, however, this precipitated portion hydrolysed readily, the rotation changing from $+50°$ to $+14{\cdot}3°$, the latter value being identical with the equilibrium rotation of *d*-mannose. The crystalline hydrolysis product from the more labile polysaccharide was identified as *d*-galactose, and that from the more stable polysaccharide was identified as *d*-mannose. By a very tedious process of fractional precipitation with alcohol a sample of the polymannose $[\alpha]_{5790} + 63°$ in water, and a sample of the polygalactose $[\alpha]_{5790} = -84°$ in water were obtained, thus showing that the original product was a mixture. The crude polymannose was a white hygroscopic powder containing approximately 15 %

of moisture and 10 % of ash, most of the latter being removed by precipitation from hydrochloric acid solution with alcohol. It gave a colourless acid solution in water and did not react with iodine or Fehling's solution. Hydrolysis with 3*N* sulphuric acid gave only mannose. On acetylation a homogeneous triacetyl compound was obtained from which the original polymannose could be regenerated with sodium hydroxide. The polygalactose was identical in appearance with the polymannose. It contained only 1·8 % of ash when crude and approximately 10 % of moisture. It was readily hydrolysed by even dilute acid to give only galactose. An ash-free triacetyl derivative was prepared which could not, however, be satisfactorily deacetylated with sodium hydroxide. The aqueous solution of the polygalactose was colourless, acid to litmus and had no action on iodine or Fehling's solution.

From Czapek-Dox medium a mixture of the same two polysaccharides was obtained. This crude mixture had a very high ash content and contained a larger proportion of the polymannose.

After precipitation of the polysaccharides with acetone, the acetone mother-liquors were evaporated *in vacuo* to remove acetone, acidified with HCl and exhaustively extracted first with ether and then with chloroform. These solvents extract a mixture of at least six new optically active acids, which have been shown to have the following empirical formulae, and for which we propose the accompanying names: $C_9H_{10}O_4$, *carolic acid*; $C_9H_{12}O_7$, *carolinic acid*; $C_{10}H_{10}O_6$, *carlic acid*; $C_{10}H_{12}O_6$, *carlosic acid*; $C_{16}H_{20}O_6$, *ramigenic acid*; $C_{26}H_{32}O_{12}$, *verticillic acid*.

Carolic acid, $C_9H_{10}O_4$, is produced in the largest yields and occurs principally in the chloroform extracts and to a much smaller extent in the ether extracts. It is produced on both media, though in larger yield on the Czapek-Dox medium. It is a dextrorotatory monobasic acid and gives a characteristic orange-yellow coloration with $FeCl_3$.

Carolinic acid, $C_9H_{12}O_7$, is the acid produced in the next largest yield to carolic acid and occurs principally in the ether extracts. The largest yields of this acid were obtained from Raulin-Thom's medium. It is a dextrorotatory dibasic acid and gives a characteristic copper salt which crystallises in pure blue needles.

Carlic acid, $C_{10}H_{10}O_6$, is produced in small amounts on both media and is present almost entirely in the chloroform extracts. It is a laevorotatory dibasic acid.

Carlosic acid, $C_{10}H_{12}O_6$, a laevorotatory dibasic acid, was obtained only on Czapek-Dox medium, none being isolated from Raulin-Thom medium. It occurs entirely in the ether extracts and was obtained in moderate yields. Carlosic acid is differentiated from the other metabolic products of *P. Charlesii* by the heavy brownish-yellow precipitate with $FeCl_3$, insoluble in excess of this reagent, by the formation on standing of a crystalline yellow precipitate with uranium acetate and by the immediate heavy precipitate, soluble in excess, which is given on careful addition of normal lead acetate.

Ramigenic acid, $C_{16}H_{20}O_6$, a dextrorotatory acid, was obtained in small yields on both media, occurring entirely in the ether extracts.

Verticillic acid, $C_{26}H_{32}O_{12}$, a laevorotatory tetrabasic acid, was obtained on both media, the larger yields being given on Raulin-Thom medium. It occurs almost entirely in the ether extracts, only traces being isolated from the chloroform extracts. A neutral aqueous solution of its sodium salt reduces silver nitrate in the cold, gives a heavy precipitate with mercuric chloride from which it can be regenerated unchanged, a definite orange-yellow colour with uranium acetate and a very intense reddish brown colour with $FeCl_3$.

In addition to the products described above, a certain amount of non-crystalline syrup has been separated. Moreover, the aqueous phase after exhaustive solvent extraction is still laevorotatory. We propose to investigate both of these fractions further.

It is the purpose of this paper to describe the cultural methods employed for the production of the substances described above, the chemical methods used for their isolation and purification and their general properties. The question of their molecular constitution will be dealt with in a series of subsequent papers.

Experimental.

Organism and culture media.

The organism used during the whole of this work was the new species of *Penicillium, P. Charlesii* G. Smith (London School of Hygiene Catalogue Number P 146). (*Cf.* Smith, 1933.)

Preliminary experiments in test-tubes showed that the reactions characteristic of *P. Charlesii* are given when the mould is grown on either the well-known Czapek-Dox solution or on the modified Raulin's solution which we have called the Raulin-Thom medium. The compositions of the two media are the following:

Czapek-Dox solution. Water, to 1000 ml.; glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.

Raulin-Thom solution. Water, to 1500 ml.; glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; diammonium hydrogen phosphate, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4, 7H_2O$, 0·07 g.; $FeSO_4, 7H_2O$, 0·07 g.

The course of metabolism.

A number of one litre conical flasks, each containing 350 ml. of Czapek-Dox solution, and a similar number of flasks of Raulin-Thom solution were sterilised and sown with a spore-suspension of *P. Charlesii* and incubated at 24°. Single flasks were taken at intervals for examination of the metabolism solution.

On Czapek-Dox solution growth was rapid but the mycelial felt was thin, the surface being rich dark green in colour and with a white reverse. The solution became very tardily pink (21 days), then brownish yellow. The rate of metabolism, as shown by analysis of the contents of single flasks, was somewhat erratic. Representative analyses are given in Table I in which are shown the

Table I.

Days of incubation	p_H	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	$FeCl_3$ reaction
0	4·5	+6·25°	5·04	Nil
3	5·1	+6·18	4·98	Nil
6	4·8	+5·72	4·61	Pale yellow
9	4·7	+5·42	4·37	Yellow
17	4·6	+4·35	3·51	Very deep yellow
25	3·4	+3·05	2·46	Orange-yellow
32	3·1	+1·73	1·40	Deep orange
51	2·4	−0·39	—	Orange-red

apparent rate of disappearance of the sugar, as determined by polarimeter (these figures are obviously somewhat inaccurate owing to the presence of optically active metabolic products), the change of reaction of the medium and the colours

obtained on addition to 5 ml. of the solution of a few drops of ferric chloride solution. An attempt was made to estimate bromine absorption by Koppeschaar's method [1876], but although it was apparent in the case of the older solutions that very considerable absorption took place, back-titration was impossible owing to rapid decomposition of the bromine compounds.

On Raulin-Thom solution growth was more rapid and the course of metabolism less erratic than on Czapek-Dox solution. The mycelial felts were comparatively thick, being bright green on the surface with a reverse which became rapidly mauve and then brownish in colour, the solution being first bright rose-pink and then brownish yellow in colour. In view of the failure to obtain satisfactory results for the estimation of bromine absorption by the Koppeschaar method an attempt was made to obtain some measure of the reactive substances produced by the mould by estimating the amount of $KMnO_4$ reduced under carefully standardised conditions. The results obtained, however, were unsatisfactory. The results obtained on the Raulin-Thom medium are given in Table II.

Table II.

Days of incubation	p_H	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	$FeCl_3$ reaction
0	3·9	+6·61°	5·34	Bright yellow
3	3·6	+6·07	4·90	Bright yellow
6	2·5	+3·64	2·94	Deep golden yellow
9	2·3	+1·90	1·53	Bright orange
11	2·2	+1·22	0·98	Bright orange
14	2·0	+0·70	0·56	Bright orange
17	2·0	+0·30	0·24	Deep orange
20	2·0	+0·11	0·09	Reddish orange
23	2·2	0	0	Reddish orange

Cultivation of P. Charlesii *in the routine metabolism experiments.*

In each of twelve experiments 100 one litre conical flasks, each containing 350 ml. of the selected medium, were sterilised by steaming on three successive days and were then sown with an aqueous spore suspension made from four cultures, 2 to 3 weeks old, grown on beer wort-agar slopes. The flasks were incubated at 24° until the sugar had completely disappeared, as evidenced by the solution becoming optically inactive or slightly laevorotatory. The duration of incubation on the Raulin-Thom medium was 24–28 days and on the Czapek-Dox medium about 8 weeks.

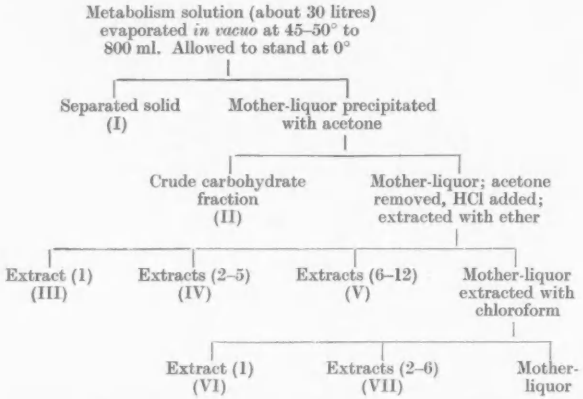
Separation of metabolic products.

At the end of the incubation period the metabolism solution was filtered from the mycelium, and the latter was well pressed and washed with a small quantity of water, the washings being added to the main filtrate. The metabolic products were first separated from the metabolism solution in a series of fractions which were eventually examined individually. The general scheme of separation of these fractions was as shown on p. 98.

This scheme relates to the experiments with Raulin-Thom medium. With Czapek-Dox medium the method of separation employed was the same but the grouping of the extracts was slightly different. Thus for Czapek-Dox medium, fraction (IV) refers to ether extracts (2–3), fraction (V) to ether extracts (4–10) and fraction (VII) to chloroform extracts (2–5).

The combined filtered metabolism solution and washings from 100 flasks were evaporated *in vacuo* at 45–50°, using the apparatus described by Raistrick and

Smith [1933], to 800 ml. and allowed to stand for 1 day at 0°. An amorphous solid separated and was filtered off, washed and dried (fraction I). To the mother-liquor, now about 1000 ml. in volume, were added 4 litres of commercial acetone, the mixture being well stirred and allowed to stand until the sticky


Metabolism solution (about 30 litres) evaporated *in vacuo* at 45–50° to 800 ml. Allowed to stand at 0°
Separated solid (I)
Mother-liquor precipitated with acetone
Crude carbohydrate fraction (II)
Mother-liquor; acetone removed, HCl added; extracted with ether
Extract (1) (III)
Extracts (2–5) (IV)
Extracts (6–12) (V)
Mother-liquor extracted with chloroform
Extract (1) (VI)
Extracts (2–6) (VII)
Mother-liquor

precipitate produced had completely settled. The clear liquid was decanted off and the precipitate washed twice with small quantities of acetone, about 200 ml. in all. The residual sticky solid was dissolved in 200 ml. of water, the solution filtered and the crude carbohydrate reprecipitated in a less sticky form by the rapid addition of 9 volumes of ethyl alcohol. The solid was separated by decantation, then ground with absolute ethyl alcohol until the whole was in the form of a powder, which was filtered off, well drained at the pump and dried in air (fraction II).

The combined aqueous acetone mother-liquors and washings were evaporated *in vacuo* to about 600 ml. in order to remove all the acetone. To the concentrate, 100 ml. concentrated HCl were added. A small amount of oily material separated at this stage but was ignored as it was difficult to separate and was readily redissolved during the subsequent extractions. The acidified solution was extracted 12 times (Raulin-Thom) or 10 times (Czapek-Dox) with its own volume of ether. Using Raulin-Thom medium, the first extract (fraction III) was kept separate and extracts 2–5 were combined as fraction IV, as were also extracts 6–12 as fraction V. With Czapek-Dox medium extracts 2–3 were combined as fraction IV and extracts 4–10 were combined as fraction V. The aqueous mother-liquor was then extracted 6 times (Raulin-Thom) or 5 times (Czapek-Dox) with its own volume of chloroform, the first extract (fraction VI) being kept separate and the remaining extracts being combined as fraction VII. All the extracts were evaporated *in vacuo* to give orange syrups which on keeping for some time gradually solidified to sticky masses of small crystals.

The aqueous solution, after extraction with chloroform, still gave a faint orange colour with ferric chloride, readily reduced $KMnO_4$ and was definitely laevorotatory. Extraction with ether or with chloroform in a continuous extractor for several days removed only very small amounts of dark-coloured sticky syrup. It is possible that the residual unextracted acid is carlic acid $C_{10}H_{10}O_6$, since its relative solubility in water, ether and chloroform would render its extraction very slow (see p. 106).

The dry weights of all the above fractions for eight typical experiments (Nos. 5 to 12), four on each medium, are summarised in Table III. The fractions from Exp. 1–4 (twelve experiments in all, six on each medium, have been carried out) were used in the preliminary work of detecting and isolating the various products present, of obtaining their solubilities and investigating their general properties.

Table III.

Exp. No.	Medium	Fractions: weight in g. I	II	III	IV	V	VI	VII
5	Czapek-Dox	10·9	53·6	30·0	35·4	40·2	51·8	25·6
6	,,	25·4	48·7	30·3	18·1	30·2	50·0	15·7
7	,,	5·9	51·6	30·0	25·6	36·0	44·0	20·5
10	,,	11·5	45·6	22·0	14·2	20·8	40·2	20·8
8	Raulin-Thom	4·0	39·7	18·0	37·9	26·8	30·4	13·6
9	,,	5·4	41·0	29·0	31·0	21·4	26·0	12·3
11	,,	4·9	43·0	19·4	26·0	19·0	24·8	11·2
12	,,	1·7	44·8	31·4	27·0	18·0	24·0	14·0

Since in each experiment 100 flasks were used containing in all 1750 g. glucose at the beginning of the experiment, and the flasks were taken off when the sugar had disappeared, the yields of these fractions in relation to the sugar utilised can readily be computed.

Isolation of products from fractions I–VII.

A. *Fraction I.*

This fraction consisted, using both media, of a yellowish-brown, amorphous powder which was insoluble in all the usual organic solvents, was non-acidic, contained no nitrogen and did not melt on heating but charred at about 250°. It was not further investigated.

B. *Fraction II.*

The following are the details of the preliminary examination of this fraction, which consisted essentially of a mixture of two polysaccharides together with mineral salts. A more detailed investigation of these polysaccharides is now in progress and will be reported on in a subsequent paper.

I. *Investigation of the polysaccharides from Raulin-Thom's medium.* The crude polysaccharide was precipitated as a red viscid mass by the addition of excess acetone to the concentrated metabolism solution. On trituration with absolute alcohol and washing with ether it was obtained as a yellowish hygroscopic powder which tended to form a glutinous mass on exposure to the atmosphere. The four batches of the material which were prepared had the following properties.

Table IV.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
8	39·7	9·5	$-41·5°$ ($c=2·31$)
9	41·0	10·3	$-39·5°$ ($c=2·17$)
11	43·0	4·0	$-38·7°$ ($c=2·07$)
12	44·8	5·7	$-40·1°$ ($c=2·68$)

The polysaccharide readily dissolved in water to give a milky yellow solution which rapidly cleared on centrifuging. It had no action on Fehling's solution or

on iodine and was acid to litmus. No pentose could be detected by the furfuraldehyde test but the solution after treatment with acid reduced Fehling's solution in the cold.

Hydrolysis of the crude polysaccharide with 0·4N H_2SO_4. A portion of the crude polysaccharide from experiment 8 containing 9·5 % ash was dissolved in 0·4*N* H_2SO_4, heated on the boiling water-bath and the rotation followed polarimetrically ($c=1·16$) with the following results.

0 minutes $[\alpha]_{5790}=-41·5°$, 5 minutes $[\alpha]_{5790}=-20·1°$, 10 minutes $[\alpha]_{5790}=0°$, 15 minutes $[\alpha]_{5790}=+27·2°$, 45 minutes $[\alpha]_{5790}=+55·9°$, 90 minutes $[\alpha]_{5790}=+60·5°$ (equilibrium value). This equilibrium value, which on correction for ash $=66·2°$, gave no definite indication of the sugar formed, and it was found that on pouring the rotation solution into absolute alcohol a polysaccharide was precipitated. This precipitate was removed by centrifuging, washed with absolute alcohol and ether and dried *in vacuo*. It was a white amorphous powder, soluble in water with an acid reaction to litmus and contained approximately 10 % of ash.

It was stable at 100° to 0·4*N* H_2SO_4 for 3 hours but was hydrolysed as follows by 3*N* H_2SO_4 at 100° ($c=0·97$ and rotation corrected for ash). 0 minutes $[\alpha]_{5790}=+48·7°$, 10 minutes $[\alpha]_{5790}=+29·9°$, 30 minutes $[\alpha]_{5790}=+15·5°$, 60 minutes $[\alpha]_{5790}=+14·3°$ (equilibrium value). This equilibrium value is very close to that of *d*-mannose $[\alpha]_{5790}^{20}=+15·2°$.

The crude polysaccharide (3·5 g.) was dissolved in 0·4*N* H_2SO_4 (50 ml.) and heated for 90 minutes until the rotation was constant. Alcohol was added to the cold solution until there was no further precipitate. This precipitate was isolated by the method described above. Yield $=1·1$ g. Ash content $=10·1$ %.

Examination of the alcoholic mother-liquors. A little water was added and the sulphuric acid neutralised with barium carbonate. The barium sulphate was filtered off and the solution concentrated *in vacuo* at 30°. The syrupy product was extracted with hot 95 % ethyl alcohol until the residue showed no reducing properties. Concentration of the alcoholic extract gave a clear colourless syrup which crystallised on scratching. Yield, 1·9 g.; M.P. crude 160°. Recrystallised from aqueous alcohol the crystals had M.P. 165° alone or in admixture with *d*-galactose; $[\alpha]_{5790}=148·7°\rightarrow 83·8°$ ($c=1·11$) after 2 days (equilibrium value for *d*-galactose $[\alpha]_{5790}^{20}=+83·6°$). The identity of the galactose was confirmed by the formation of galactosazone (M.P. and mixed M.P. 196°) and by the preparation, on oxidation with nitric acid, of *d*-mucic acid (M.P. 214°); yield, 77 % theoretical. No other sugar could be detected.

Hydrolysis of the more stable polysaccharide. This polysaccharide (1·1 g.) was heated for 90 minutes at 100° with 50 ml. of 3*N* sulphuric acid. The acid was neutralised with barium carbonate, the barium sulphate filtered off and the solution concentrated *in vacuo* at 30°. The syrup was extracted with 95 % alcohol, the removal of which by evaporation gave a colourless product.

On treatment of a portion of the syrup (0·2 g.) with phenylhydrazine acetate (0·2 g.) in the cold for ½ hour a sandy crystalline powder separated. After standing for an hour the crystals were filtered off and recrystallised from 50 % alcohol (0·28 g.), from which solvent they separated in the characteristic form of mannosephenylhydrazone and had M.P. and mixed M.P. with an authentic specimen 196°.

This phenylhydrazone, on heating with an excess of phenylhydrazine acetate at 100° for 5 minutes, yielded glucosazone identical with an authentic specimen.

The remainder of the syrup was scratched with a nucleus of *d*-mannose and crystallised completely in a few days, M.P. 135° (M.P. of mannose 132–133°).

Fractionation of the mixture of the two polysaccharides. It was found that the crude polysaccharide could be separated into various fractions by the graded addition of absolute alcohol to a concentrated aqueous solution. In a typical experiment 78 g. of crude polysaccharide $[\alpha]_{5790} = -41{\cdot}0°$ gave the following results.

Table V.

Fraction	$[\alpha]_{5790}$	% alcohol	% ash	Yield (g.)
1	0	50	40·3	12
2	−34·2°	66	3·5	40
3	−37·6°	80	5·8	9
4	−56·7°	Concentrated mother-liquor	3·7	14

Most of the ash was removed in the first fraction and it was found that the ash could be further separated as follows. 43 g. of crude polysaccharide with a high ash content were dissolved in 150 ml. of water. To this syrup, pyridine (200 ml.) was carefully added. A fraction (7 g.) was precipitated and removed by centrifuging. This fraction contained 90 % of ash. The remaining fractions could be precipitated by the addition of alcohol to the aqueous pyridine solution.

By an elaborate and tedious method of repeated precipitations it was possible to isolate specimens of a polymannose having $[\alpha]_{5790} = +63°$ (15 g.) and a polygalactose $[\alpha]_{5790} = -84°$ (12 g.). A series of intermediate fractions is in process of separation.

Properties of the purified polymannose. This compound was a pure white hygroscopic powder containing, before final purification, 15 % of moisture and 10 % of ash. The ash content was lowered to 2·5 % after one precipitation from weak hydrochloric acid solution by the addition of alcohol. Its aqueous solution was colourless, acid to litmus, non-reducing and without action on iodine. It was stable to N H_2SO_4 at 80° for 2 hours but was readily hydrolysed with $3N$ H_2SO_4 at 100°. A sample having an ash content of 0·7 % and dried to constant weight gave the following results on hydrolysis with $3N$ H_2SO_4 at 100°:

0 minutes $[\alpha]_{5790} = +63{\cdot}0°$, 10 minutes $[\alpha]_{5790} = +49{\cdot}1°$, 25 minutes $[\alpha]_{5790} = +31{\cdot}4°$, 45 minutes $[\alpha]_{5790} = +17{\cdot}7°$, 60 minutes $[\alpha]_{5790} = +15{\cdot}7°$, 90 minutes $[\alpha]_{5790} = +15{\cdot}7°$. Therefore the equilibrium rotation calculated as hexose $= +14{\cdot}1°$ (equilibrium rotation recorded for mannose $= +15{\cdot}2°$). Mannose was the only hydrolysis product isolated.

An analysis was carried out on the incompletely dried polysaccharide and the moisture content estimated separately. The analysis was not entirely satisfactory owing to the hygroscopic nature of the ash.

(Found: Moisture, 15·0 %; ash, 9·6 %; C, 44·6; H, 7·5 %. $(C_6H_{10}O_5)_n$ requires: C, 44·4; H, 6·2 %.)

Preparation of an acetate. The polysaccharide (1 g.) was dissolved in water (1 ml.) and pyridine (25 ml.) added slowly. A small amount of inorganic material separated and was removed by centrifuging. Acetic anhydride (25 ml.) was now added slowly and the solution maintained at 40° during the addition (10 minutes). It was then cooled to 20° and left overnight. Next morning the clear pale yellow solution was poured into 200 ml. of warm water, and the acetate was obtained as a flocculent precipitate. It was centrifuged and washed repeatedly with warm water until free from the acetylation mixture. It was dried *in vacuo*. Yield, 1·6 g. Ash content, 0·9 %. OAc, 46·1 %. $[\alpha]_{5790} = +52{\cdot}6°$ in acetone

($c=1{\cdot}56$). Fractional precipitation of the acetate from acetone by means of ether showed that it was homogeneous.

Regeneration of the polysaccharide. The acetate (1 g.) was dissolved in acetone and a slight excess of *N* NaOH added. The product was precipitated twice from weak HCl solution, washed with absolute alcohol and dried *in vacuo*. Ash content, 0·7 %. $[\alpha]_{5790}=+62°$ ($c=1{\cdot}0$).

Properties of the purified polygalactose. This was a white hygroscopic powder containing 1·83 % of ash and 10 % of moisture; $[\alpha]_{5790}=-84{\cdot}3°$. It was analysed in the same manner as the polymannose and again the ash content prevented a satisfactory analysis.

(Found: C, 44·4; H, 7·0 %. $(C_6H_{10}O_5)_n$ requires: C, 44·4; H, 6·2 %.)

The solution in water was colourless, faintly acid to litmus and with no action on Fehling's solution or on iodine.

It readily hydrolysed as follows with 0·3*N* sulphuric acid. ($c=0{\cdot}711$.) 0 minutes $[\alpha]_{5790}=-84{\cdot}3°$, 10 minutes $[\alpha]_{5790}=-50{\cdot}0°$, 15 minutes $[\alpha]_{5790}=0°$, 20 minutes $[\alpha]_{5790}=+30{\cdot}0°$, 45 minutes $[\alpha]_{5790}=+72{\cdot}0°$, 60 minutes $[\alpha]_{5790}=+88{\cdot}0°$ (equilibrium value). Equilibrium value corrected for ash and as hexose $=+80{\cdot}8°$; $[\alpha]^{20}_{5790}$ for *d*-galactose $=+83{\cdot}6°$.

Acetylation. The polysaccharide (1 g.) was acetylated and the product isolated in the manner described for the polymannose. Yield, 1·5 g. of a white, ash-free powder. It had $[\alpha]_{5790}$ in acetone $=-72{\cdot}8°$ ($c=0{\cdot}75$). Acetyl content, 44·4 %. (Theoretical for the triacetate of a polyhexose, $C_6H_7O_2(OCOCH_3)_3$, 44·8 %.)

Regeneration of the polysaccharide by deacetylation with sodium hydroxide was unsatisfactory, the product being extremely hygroscopic.

II. *Investigation of the polysaccharides from Czapek-Dox medium.* The products were obtained by precipitation by acetone from the concentrated metabolism solution and isolated as previously described for Raulin-Thom medium. The ash content was very high (approximately 30 %), and the product was more soluble in water giving a clear yellow solution. The yields and properties were as follows.

Table VI.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
5+6	102·3	30·0	0
7	51·6	31·2	−8·0°
10	45·6	28·5	−5·0°

Fractional precipitation from aqueous solution with alcohol removed most of the ash in the first fraction, and a series of fractions with different rotations was obtained. The crude material behaved on hydrolysis in a manner exactly similar to the product described from Raulin-Thom medium.

Hydrolysis of the crude polysaccharide with 0·5N H_2SO_4. A portion of the crude polysaccharide containing 9·5 % ash, and having an initial rotation of $[\alpha]_{5790}=-15{\cdot}3°$ dissolved ($c=0{\cdot}96$) in 0·5*N* H_2SO_4, and heated at 100° reached an equilibrium value of $[\alpha]_{5790}=+48°$ in 70 minutes.

Strong sulphuric acid was now added to make a 3*N* solution and the solution heated again at 100°. The rotation fell to an equilibrium value of $[\alpha]_{5790}=+35{\cdot}3°$ in 60 minutes.

The crude polysaccharide (3 g.) was hydrolysed with 0·3*N* H_2SO_4 as previously described. 2 g. of unhydrolysed polysaccharide were obtained on pouring the solution into absolute alcohol. This polysaccharide gave *d*-mannose on hydrolysis and after purification was identical with the polymannose previously described.

The mother-liquors on concentration and extraction with alcohol gave crystalline *d*-galactose, and it is probable that the crude polysaccharide is a mixture of the same polymannose and polygalactose as from the Raulin-Thom medium though this mixture has a higher polymannose content.

C. *Fractions III, IV and V (Ether extracts).*

Each of the above fractions, representing the material extracted by ether in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of chloroform (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with chloroform. In this way, each extract was separated into a quantity of white or light brown crystalline material and a chloroform mother-liquor which, after removal of chloroform, was dried down to a reddish brown syrup.

Using Czapek-Dox medium, the crystalline material obtained from fractions III and IV consisted of almost pure carlosic acid and from fraction V of almost pure carolinic acid, the total yields of which are given in Table VII. It appears therefore, that carlosic acid is completely extracted in the first three of the ten ether extracts, together with small amounts of carolinic acid, whilst the latter acid is extracted more slowly and is accumulated in the remaining ether extracts. This behaviour may be readily accounted for by considering the relative solubilities of these two acids in water and ether as shown later.

Using Raulin-Thom medium the crystalline material consisted in each of the fractions III, IV and V of a mixture of carolinic acid with varying but smaller amounts of verticillic acid, the total yields of which are given in Table VII. These two acids were then separated by fractionation with acetone in which verticillic acid is very much less soluble than carolinic acid.

Examination of the syrups obtained by drying down the chloroform mother-liquors from the above acids showed that in all cases appreciable amounts of carolic acid are present. In order to separate this acid, the combined syrups from experiments 5, 6, 7 and 10 on Czapek-Dox medium and, separately, the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium were thoroughly triturated with water. The combined syrups from the Czapek-Dox medium were extracted with a total volume of 450 ml. of water and the syrups from the Raulin-Thom medium with 300 ml. of water.

The aqueous extracts were then filtered and extracted once with an equal volume of chloroform. The chloroform extracts were evaporated and the resultant yellowish oil seeded with carolic acid and set aside to crystallise. The crystalline residue was finally rubbed up with a little absolute ethyl alcohol, filtered and washed with a small amount of alcohol, when almost pure carolic acid resulted. By this treatment the combined syrups from the experiments 5, 6, 7 and 10 on Czapek-Dox medium (total weight after drying at 50° *in vacuo* = 250 g.) yielded 53·5 g. of yellowish oil from which 30 g. of carolic acid were obtained, while the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium (total weight after drying at 50° *in vacuo* = 200 g.) yielded 35·4 g. of yellowish oil from which 18 g. of carolic acid were obtained.

Having removed the carolic acid from the combined syrups by water extraction as described above, the water-insoluble portion of the syrups was allowed to stand for four weeks when further crystallisation occurred, and small amounts of ramigenic acid separated in hard, well-formed prisms. These were filtered off, washed clean from syrup by rubbing up with chloroform and recrystallised

from acetone-light petroleum. The weights of pure ramigenic acid obtained from the syrups from the Raulin-Thom and the Czapek-Dox media were 3 g. and 1 g. respectively.

The syrups remaining after separation of ramigenic acid still contained small amounts of verticillic acid, amounting to about 7 % of the weight of the syrup from both media. In addition, in the case of the syrup from Czapek-Dox medium, but not from Raulin-Thom medium, a very small amount of carlosic acid was detected. These two acids were isolated by making use of specific precipitating reagents. Thus verticillic acid was separated by neutralising an aliquot portion of the syrup with sodium hydroxide to phenolphthalein and adding mercuric chloride which, as will be shown later, precipitates verticillic and ramigenic acids but does not precipitate the remaining four acids. The mercury precipitates from the two media were then suspended in water, the mercury removed with hydrogen sulphide, the excess of hydrogen sulphide removed in a stream of air and the solutions extracted with ether. After removal of the ether, the extracted material crystallised and was rubbed up with a small volume of acetone, filtered and washed with a little acetone. The residual crystalline material was shown by comparison with an authentic specimen to be verticillic acid. Finally, the presence of a very small amount of carlosic acid in the syrups from Czapek-Dox medium was detected by carefully adding normal lead acetate to the neutralised solution of an aliquot portion of the syrup. This reagent, as will be shown later, specifically precipitates carlosic acid, but its addition requires care as the precipitate redissolves in excess of the reagent. The lead precipitate was then rubbed up with $2N$ H_2SO_4 and the carlosic acid extracted with ether. After removal of the ether, the yellow crystalline mass was rubbed up with chloroform, filtered and washed with a little chloroform. The very small amount of crystalline material was shown by comparison with an authentic specimen to be carlosic acid $C_{10}H_{12}O_6$.

D. *Fractions VI and VII (Chloroform extracts).*

Each of the above fractions, representing the material extracted by chloroform in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of absolute ethyl alcohol (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with a little alcohol. In this way all the fractions VI gave a white crystalline powder consisting of almost pure carolic acid and amounting to 70 % of the total weight of the fractions, whilst all the fractions VII gave small yields of a white crystalline powder consisting of fairly pure carlic acid. The alcoholic mother-liquors from fractions VI and VII were evaporated *in vacuo* and extracted with water in which they were almost completely soluble. The aqueous solutions

Table VII. *Yields in g. of isolated and almost pure crystalline acids.*

Exp. No.	Medium	Carolic acid $C_9H_{10}O_4$	Carolinic acid $C_9H_{12}O_7$	Carlic acid $C_{10}H_{10}O_6$	Carlosic acid $C_{10}H_{12}O_6$	Verticillic acid $C_{26}H_{22}O_{12}$	Ramigenic acid $C_{16}H_{20}O_6$	
5	Czapek-Dox	44·7	8·2	7·5	9·3	6·7		
6	,,	43·7	10·2	5·4	9·0	4·4		1 g.
7	,,	44·6	3·9	1·7	5·6	5·9		
10	,,	42·4	7·8	5·3	9·6	3·3		
8	Raulin-Thom	29·7	16·3	4·1	—	13·6		
9	,,	27·9	14·9	3·0	—	13·2		3 g.
11	,,	29·6	14·5	1·3	—	11·5		
12	,,	26·7	16·6	1·1	—	4·2		

were then extracted once with an equal volume of chloroform, the chloroform removed and the partially crystalline residue washed with alcohol, further small amounts of carolic acid being thus obtained. The total yield of carolic acid obtained from fraction VI was thus raised to 75 % of the weight of this fraction.

The final yields of the practically pure crystalline acids in the eight typical experiments are given in Table VII.

Properties and analysis of the crystalline acids.

We propose now to give details of the general properties and analysis of the different crystalline acids isolated, reserving for future communications the question of their molecular constitution. The equivalents were determined by dissolving a weighed amount in a suitable solvent and titrating to phenolphthalein with $N/10$ sodium hydroxide. Optical rotations were determined at room temperature on the free acids in a suitable solvent and also on neutral aqueous solutions of the sodium salts using the mercury green line λ_{5461}. Solubilities were determined roughly by shaking 0·1 g. of the finely powdered material at room temperature with a gradually increasing and measured amount of the solvent in question until dissolution was just complete. The reactions of aqueous solutions of the sodium salts of the different acids towards a series of inorganic reagents were determined by adding to 2 ml. of a 1 % aqueous solution of the acid, neutralised to phenolphthalein with sodium hydroxide, 1 ml., drop by drop, of a 10 % aqueous solution of the reagent in question. All micro-analyses were carried out by Schoeller (Berlin).

Carolic acid $C_9H_{10}O_4$. Carolic acid is best purified by recrystallising (2 g.) from boiling ethyl alcohol (20 ml.). Yield 1·4 g. of colourless radiating needles, M.P. 132° without obvious decomposition. On resetting and remelting the M.P. gradually falls to a constant value of 113°.

(Found: C, 59·45, 59·42; H, 5·61, 5·58 %; OCH_3, nil; Mol. Wt. 217, 235. $C_9H_{10}O_4$ requires: C, 59·31; H, 5·54 %; Mol. Wt. 182.) The molecular weight found by the depression of the freezing-point of benzene was 196.

Titration. 0·2380 g. and 0·2318 g. required respectively 13·05 and 12·70 ml. $N/10$ NaOH, corresponding to equivalents of 182·4 and 182·5 respectively. ($C_9H_{10}O_4$ titrating as a monobasic acid requires 182·1.)

Rotations. (*a*) Free acid in water: ($c = 0·50$) $[\alpha]_{5461} = +84°$.

This value remained constant for 1 hour but had fallen after 24 hours to +81°; the solution becoming faintly yellow in colour.

(*b*) Sodium salt: ($c = 0·52$ calculated as monosodium salt) $[\alpha]_{5461} = +71·5°$.

This value did not change overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 2 ml. chloroform, 12 ml. water or acetone, 28 ml. ethyl acetate, 36 ml. ethyl alcohol, 40 ml. benzene, 400 ml. ether, but was not completely dissolved in 500 ml. light petroleum, B.P. 40–50°.

Salts. Carolic acid gives no precipitate even on standing overnight with mercuric chloride, copper sulphate, uranium acetate, normal or basic lead acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate gives no precipitate even in concentrated solution and no reduction on standing overnight. Ferric chloride gives a characteristic bright orange colour but no precipitate (*cf.* carolinic acid).

An aqueous solution of the acid is strongly acid to Congo red, reduces acid or neutral permanganate immediately in the cold and decolorises bromine water instantly. With Brady's reagent (2 : 4-dinitrophenylhydrazine in 2 *N* HCl), there is no immediate precipitate but on standing with excess of reagent a crystalline orange precipitate begins to separate after 48 hours and continues for 3 to 4 weeks. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, remaining colourless at 100° but at 140–150° becoming first rose-pink, then darkening to a characteristic intense bluish red colour.

Carolinic acid $C_9H_{12}O_7$. ($C_9H_{10}O_6$, H_2O.) Carolinic acid is best purified by recrystallisation from acetone (or acetone-light petroleum) from which solvent it separates in colourless prisms, M.P. 123° with evolution of gas.

(Found: C, 46·53, 46·43; H, 5·26, 5·21 %; OCH_3, nil. $C_9H_{12}O_7$ requires: C, 46·53; H, 5·21%; Mol. Wt. 232.) The molecular weight of carolinic acid must be that corresponding to the formula $C_9H_{12}O_7$ since it readily loses $1H_2O$ giving a product having an observed molecular weight corresponding to $C_9H_{10}O_6$ (see below).

Titration. 0·1723 g. required 14·50 ml. *N*/10 NaOH corresponding to an equivalent of 117·. ($C_9H_{12}O_7$ titrating as a dibasic acid requires 116.)

Rotation. (*a*) Free acid in water: ($c = 0·34$) $[\alpha]_{5461} = +60°$. The rotation did not change overnight but the solution became faintly yellow in colour.

(*b*) Disodium salt: ($c = 0·41$; calculated as the disodium salt) $[\alpha]_{5461} = +54°$. The rotation did not change overnight and the solution remained colourless.

Solubilities. 1 g. dissolves in 25 ml. ethyl alcohol, 30 ml. acetone, 150 ml. water, 170 ml. ethyl acetate, but was not completely dissolved in 500 ml. benzene, chloroform, ethyl ether or light petroleum, B.P. 40–50°.

Salts. Carolinic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brown precipitate, soluble in excess to a bright orange-coloured solution. Copper sulphate gives no immediate precipitate but on standing there is a copious separation of beautiful pure blue needles. Basic lead acetate gives a very heavy immediate precipitate completely soluble in excess. Barium or calcium chloride in presence of four volumes of ethyl alcohol gives a gelatinous precipitate tending to become crystalline on standing.

An aqueous solution of the acid is strongly acid to Congo red and only slowly reduces permanganate in the cold. With Brady's reagent it slowly gives a slight yellow precipitate. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes dark brownish red on heating to 140–150°.

Carolinic acid, crystallised from acetone, readily loses a molecule of water on drying at 80°. 0·2216 g. dried to constant weight at 80° lost 0·0170 g. $H_2O = 7·67$ % H_2O. (Theoretical for $C_9H_{12}O_7 \rightarrow C_9H_{10}O_6 = 7·76$ %.)

(Found for the dried substance: C, 50·81; H, 4·73 %; Mol. Wt. 223, 231; equivalent, 105. $C_9H_{10}O_6$ requires: C, 50·45; H, 4·71 %; Mol. Wt. 214; equivalent titrating as a dibasic acid, 107.) The dehydrated material on recrystallisation from acetone reverts to carolinic acid $C_9H_{12}O_7$.

Carlic acid $C_{10}H_{10}O_6$. Carlic acid is best purified by recrystallising from ethyl alcohol, from which it separates in colourless needles, M.P. 176°, giving a brown gummy melt which does not resolidify on cooling.

(Found: C, 53·23, 53·22; H, 4·53, 4·47 %; OCH_3, nil. $C_{10}H_{10}O_6$ requires: C, 53·08; H, 4·46 %; Mol. Wt. 226.) The molecular weight found by the depression of the freezing-point of dioxan was 240. The empirical formula of carlic acid $C_{10}H_{10}O_6$ also follows from that of carolic acid $C_9H_{10}O_4$ since, from observations which will be published later, it is known that the molecule of carlic acid contains CO_2 more than that of carolic acid.

Titration. 0·1214 g. required 10·70 ml. *N*/10 NaOH corresponding to an equivalent of 113·4. ($C_{10}H_{10}O_6$ titrating as a dibasic acid requires 113.)

Rotation. (*a*) Free acid in water: ($c = 0·28$) $[\alpha]_{5461} = -160°$. This value was not changed overnight and the solution remained colourless.

(*b*) Disodium salt: ($c = 0·29$; calculated as the disodium salt) $[\alpha]_{5461} = -147°$. This value was not changed overnight and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. water, 45 ml. acetone, 75 ml. ethyl alcohol, 270 ml. ethyl acetate, 350 ml. chloroform, 500 ml. benzene, but was not completely dissolved in 500 ml. ethyl ether, light petroleum, B.P. 40–50°, or butyl alcohol.

Salts. Carlic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brownish-yellow precipitate soluble in excess to an orange-yellow solution. Copper

sulphate gives no immediate precipitate but after several days' standing blue-green nodular crystals separate, which are quite different in colour from those given by carolinic acid. Basic lead acetate gives a very heavy precipitate which is readily soluble in excess. Calcium chloride in presence of four volumes of alcohol does not give an immediate precipitate, but on standing for several days a moderate precipitate is formed. Barium chloride in presence of four volumes of alcohol gives an immediate heavy flocculent precipitate.

An aqueous solution is acid to Congo red, reduces acid permanganate fairly slowly and does not give a precipitate with Brady's reagent. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, which becomes only pale brown on heating for some time at 140–150°.

Carlosic acid $C_{10}H_{12}O_6$. Carlosic acid is best purified by crystallisation from a large volume of boiling benzene from which it separates in fine colourless needles, M.P. 181°.

(Found: C, 52·51, 52·62; H, 5·35, 5·30 %; OCH_3, nil; Mol. Wt. 236, 242. $C_{10}H_{12}O_6$ requires: C, 52·61; H, 5·31 %; Mol. Wt. 228.)

Titration. 0·1040 g. required 9·20 ml. *N*/10 NaOH corresponding to an equivalent of 113·1. ($C_{10}H_{12}O_6$ titrating as a dibasic acid requires 114.)

Rotations. (*a*) Free acid in water: ($c = 0·21$) $[\alpha]_{5461} = -160°$. This value was unchanged overnight and the solution did not become coloured.

(*b*) Disodium salt: ($c = 0·25$ calculated as the disodium salt) $[\alpha]_{5461} = -150°$. This value was unchanged overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 14 ml. acetone, 30 ml. ethyl alcohol, 45 ml. ethyl acetate, 90 ml. water, 250 ml. of chloroform or ether, 500 ml. benzene, but was not completely dissolved in 500 ml. light petroleum, B.P. 40–50°.

Salts. Carlosic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, copper sulphate or calcium chloride in presence of four volumes of ethyl alcohol. Ferric chloride gives an immediate brownish-yellow precipitate insoluble in excess. Uranium acetate gives no immediate precipitate but on standing for 2–3 days a characteristic copious semi-crystalline yellow precipitate separates. Both normal and basic lead acetates give immediate very heavy precipitates soluble in large excess. Barium chloride in presence of four volumes of alcohol gives a flocculent white precipitate.

An aqueous solution is acid to Congo red, reduces acid $KMnO_4$ fairly slowly, and gives an immediate orange-yellow crystalline precipitate with Brady's reagent, M.P. 135°. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes only pale brown on heating at 140–150°.

Ramigenic acid $C_{16}H_{20}O_6$. Ramigenic acid is precipitated on acidifying aqueous solutions of the sodium salt in large prisms containing $1H_2O$ (*a*), which is lost on standing *in vacuo* over P_2O_5 for several days. It crystallises in well-formed prisms from acetone-light petroleum (*b*). The acid $C_{16}H_{20}O_6, H_2O$ softens slightly at 125° owing to loss of water, but does not melt. The anhydrous acid melts at 171°.

(Found: (*a*) C, 59·24, 59·10; H, 6·62, 6·75 %; OCH_3, nil. $C_{16}H_{20}O_6, H_2O$ requires: C, 58·86; H, 6·80 %. (*b*) C, 62·40, 62·30; H, 6·59, 6·51 %; OCH_3, nil. $C_{16}H_{20}O_6$ requires: C, 62·30; H, 6·54 %.)

Titration. 0·2343 g. of the acid $C_{16}H_{20}O_6$ was dissolved in 20 ml. absolute alcohol and titrated to phenolphthalein. The first pink flush (rapidly disappearing) was obtained after adding 7·70 ml. *N*/10 NaOH corresponding to an equivalent of 304 ($C_{16}H_{20}O_6$ titrating as a monobasic acid requires 308). A further 12·30 ml. *N*/10 NaOH (total 20·0 ml.) were added and the solution heated on the water-bath for 2 hours. On cooling and titrating, the red colour first disappeared after adding 5·24 ml. *N*/10 H_2SO_4, corresponding to an equivalent of 158·8 ($C_{16}H_{20}O_6$ titrating as a dibasic acid requires 154). On standing, the solution became slowly pink, was decolorised by addition of a few drops of *N*/10 H_2SO_4 and, on standing, again became pink. The process was repeatable, indicating that the more difficultly opened acidic group responsible for the dibasic character closes with great ease in more acid solution. The solution was then acidified and allowed to stand. Well-formed prisms having the characteristics of ramigenic acid separated, M.P. 171° (after loss of water at 125°) and not depressed on mixing with an authentic sample.

Rotations. (*a*) Free acid in alcohol: ($c = 0{\cdot}22$) $[\alpha]_{5461} = +28°$. The rotation did not change overnight, and the solution did not become coloured.

(*b*) Sodium salt: ($c = 0{\cdot}27$; calculated as monosodium salt) $[\alpha]_{5461} = +85°$. The rotation did not change overnight, and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. acetone, 16 ml. ethyl alcohol, 30 ml. chloroform, 90 ml. ethyl acetate, 180 ml. ether, but was not completely dissolved in 500 ml. water, benzene or light petroleum, B.P. 40–50°.

Salts. Ramigenic acid gives no precipitate with copper sulphate, uranium acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate is slowly reduced in the cold, giving a silver mirror on standing overnight. Ferric chloride gives a heavy pale brown crystalline precipitate, probably of ramigenic acid, the supernatant liquid being only light yellowish brown in colour. Mercuric chloride gives an immediate yellow colour with slow development of a heavy pale yellow precipitate. Normal and basic lead acetates give slight white precipitates.

The acid is insoluble in water and an aqueous suspension is not acid to Congo red and reduces $KMnO_4$ immediately in neutral and more slowly in acid suspension. No reaction occurs with Brady's reagent, but this may be due to the insolubility of the acid. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

Verticillic acid $C_{26}H_{32}O_{12}$. Verticillic acid is best purified by allowing the boiling saturated acetone solution to stand overnight, when the acid separates in fine needles, M.P. 171°, giving a brown viscous melt which does not reset on cooling.

(Found: C, 58·06, 58·24; H, 6·04, 6·06 %; OCH_3, nil. $C_{26}H_{32}O_{12}$ requires: C, 58·17; H, 6·02 %.)

Titration. Verticillic acid on titrating in alcoholic solution behaves like an acid lactone; 0·1578 g. dissolved in 20 ml. alcohol gave the first pink flush after the addition of 11·09 ml. *N*/10 NaOH. The titration then became slow as for a lactone. After standing for three hours at room temperature with an excess of NaOH, the final titration was 11·74 ml. *N*/10 NaOH, corresponding to an equivalent of 134·5 ($C_{26}H_{32}O_{12}$ titrating as the lactone of a tetrabasic acid requires 134).

Rotations. (*a*) Free acid in alcohol: ($c = 0{\cdot}40$) $[\alpha]_{5461} = -53°$. This value rapidly fell on standing and the solution became progressively more and more yellow in colour. The specific rotations after 10, 15 and 45 minutes, 2, 5, 24, 48 and 72 hours were respectively $-47°$, $-44°$, $-36°$, $-35°$, $-34°$, $-28°$, $-20°$, $-14°$.

(*b*) Sodium salt: ($c = 0{\cdot}49$, calculated as the tetrasodium salt) $[\alpha]_{5461}$ initially $= +46°$ and only slowly decreases to $+41°$ after 24 hours, the solution becoming faintly yellow in colour.

Solubilities. 1 g. dissolved in 45 ml. ethyl alcohol, 270 ml. acetone, but was not completely dissolved in 500 ml. water, benzene, chloroform, ethyl acetate, ether and light petroleum, B.P. 40–50°.

Salts. Silver nitrate gives no precipitate, but reduction commences after 2–3 hours in the cold with the formation of a silver mirror overnight. Mercuric chloride gives an immediate heavy white precipitate with a yellow supernatant fluid. Ferric chloride gives a characteristic very intense brownish-red colour. Uranium acetate gives no precipitate but a characteristic orange-yellow colour. Slight precipitates are given with copper sulphate, normal and basic lead acetates and calcium or barium chloride in presence of four volumes of alcohol.

An aqueous solution of verticillic acid is faintly acid to Congo red and reduces acid permanganate very readily in the cold. An alcoholic solution of the acid gives no immediate precipitate with Brady's reagent, but an orange precipitate begins to separate after 12 hours. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

The molecular weights by the Rast camphor method for both verticillic and ramigenic acids were unsatisfactory, owing to the fact that they both decompose on heating at a temperature below that of the melting-point of camphor. The molecular formulae $C_{26}H_{32}O_{12}$ and $C_{16}H_{20}O_6$ have been assigned to verticillic and ramigenic acids respectively for the following reasons:

(*a*) Verticillic acid (1 molecule) on mild hydrolysis gives rise to 1 molecule of ramigenic acid and a keto-acid which analyses for the empirical formula of $(C_5H_6O_3)_n$.

(*b*) Ramigenic acid titrates in the cold as a monobasic acid, equivalent 304 ($C_{16}H_{20}O_6$ requires 308).

(*c*) Ramigenic acid with diazomethane gives a crystalline monomethyl derivative containing 9·60 % OCH_3 and having an observed molecular weight by the Rast camphor method of 332 and 340 ($C_{16}H_{19}O_5OCH_3$ requires 9·63 % OCH_3 and molecular weight 322). It follows therefore that the empirical formula for ramigenic acid must be $C_{16}H_{20}O_6$ and hence that for verticillic acid is $C_{26}H_{32}O_{12}$.

Summary and discussion.

The metabolic products of a new mould species isolated from mouldy Italian maize, *Penicillium Charlesii* G. Smith, have been investigated. This mould was grown at 24° on Czapek-Dox medium in which glucose is the only source of carbon, and on Raulin-Thom medium in which glucose is the only carbohydrate present. The metabolic products isolated consist of two new polysaccharides and six hitherto undescribed optically active organic acids.

The polysaccharides are a polygalactose $[\alpha]_{5790} = -84°$ and a polymannose $[\alpha]_{5790} = +63°$ which give on hydrolysis *d*-galactose and *d*-mannose respectively as the only carbohydrates. Both of these polysaccharides were formed on both media and their production in considerable amounts affords an interesting example of the ready conversion by a mould of glucose into mannose and galactose.

The six organic acids isolated are as follows: (*a*) carolic acid $C_9H_{10}O_4$, (*b*) carolinic acid $C_9H_{12}O_7$, (*c*) carlic acid $C_{10}H_{10}O_6$, (*d*) carlosic acid $C_{10}H_{12}O_6$, (*e*) ramigenic acid $C_{16}H_{20}O_6$ and (*f*) verticillic acid $C_{26}H_{32}O_{12}$. Of these (*a*), (*b*) and (*e*) are dextrorotatory while (*c*), (*d*) and (*f*) are laevorotatory. The acids were isolated from the metabolism solution and separated from each other in a state of purity by taking advantage of their differing solubilities and of their varying precipitability by different metallic salts.

It is a point of considerable microbiological interest to compare the products of metabolism of three species of *Penicillium*, all of which were isolated from mouldy maize, *i.e.* of *P. brevi-compactum* Dierckx (syn. *P. stoloniferum* Thom), *P. Charlesii* G Smith, each of which was isolated from mouldy Italian maize, and *P. puberulum* Bainier from mouldy American maize. *P. brevi-compactum* produces from glucose the following series of optically inactive phenolic acids, each containing a benzenoid nucleus: $C_{17}H_{20}O_6$, $C_{10}H_{10}O_7$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_5$ and $C_8H_6O_6$ [Clutterbuck *et al.*, 1932; Oxford and Raistrick, 1932; 1933, 1, 2; Clutterbuck and Raistrick, 1933]. *P. Charlesii* on the other hand produces from glucose the following series of optically active non-benzenoid acids; $C_{26}H_{32}O_{12}$, $C_{16}H_{20}O_6$, $C_{10}H_{12}O_6$, $C_{10}H_{10}O_6$, $C_9H_{12}O_7$ and $C_9H_{10}O_4$. It will be noticed that two acids having the empirical formula $C_{10}H_{10}O_6$, but differing widely in chemical constitution, are produced by *P. brevi-compactum* and *P. Charlesii*, which curiously enough were isolated from the same sample of mouldy Italian maize. *P. puberulum* produces from glucose three organic acids, penicillic acid $C_8H_{10}O_4$ [Alsberg and Black, 1913], puberulic acid $C_8H_6O_6$, and an acid $C_8H_4O_6$ [Birkinshaw and Raistrick, 1932]. Each of these three acids is optically inactive and non-benzenoid in nature.

In view of the amazing variety of the metabolic products of different species of Penicillia occurring in mouldy maize, some of which are known to be definitely toxic, it is tempting to enquire whether the possibility has been entirely ruled out that mould infection of maize has a connection with human pellagra. Most workers on human pellagra appear to agree that this disease is intimately connected in some way with the consumption of maize. Aykroyd [1933] divides the views which have been expressed as to the origin of pellagra under three headings:

(*a*) theory of a maize toxin,

(*b*) theory of amino-acid deficiency due to the low biological value of maize proteins,

(*c*) theory of vitamin deficiency.

It would be outside the scope of this paper to discuss (*b*) and (*c*), but we venture to raise the question as to whether the presence of toxic materials elaborated by organisms infecting maize may not form a contributory factor. In this connection it is of interest to note, to quote Aykroyd (page 343), that "Italian antipellagra policy is enshrined as a law passed in 1902 which was based on the theory of causation generally accepted at that date. The sale of 'spoiled' (*i.e.* infected) maize as human food was strictly prohibited, and local authorities were forced to acquire public desiccators and municipal reservoirs in which maize could be kept in good condition." This is particularly interesting in view of the fact that the death rate per 100,000 of population from pellagra reported officially in Italy was 10·7 in the period 1896–1900, a figure which steadily decreased to 0·2 in 1928. On the other hand, in the United States of America where, so far as we are aware, no such law is in force as exists in Italy, the death rate per 100,000 in 1924 was 2·5, increasing steadily to 6·4 in 1928 and being 5·9 in 1930.

We desire to express our sincere thanks to our colleague Dr A. E. Oxford for the determination of the molecular weights of carolic and carlic acids, and to Mr W. K. Anslow for much technical assistance during the course of this work.

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XVII. THE VITAMIN D ACTIVITY OF BUTTER. II. THE COMPLEX NATURE OF THE ANTIRACHITIC ACTION OF BUTTER.

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(*Received January 1st, 1934.*)

In the first paper of this series [Kon and Booth, 1933, 2] evidence was brought forward to show that the antirachitic activity of autumn and winter butter cannot be quantitatively recovered in the non-saponifiable residue of these butters. Under exactly similar experimental conditions irradiated ergosterol or cod-liver oil could be subjected to saponification either alone or mixed with winter butter without loss of potency, *i.e.* their non-saponifiable residues carried the whole of their antirachitic activities.

Since then we have studied the effect of saponification on butters produced under summer conditions and also on butters rendered highly antirachitic either by direct irradiation with the quartz mercury vapour lamp, or by the feeding to cows of large amounts of irradiated yeast or of cod-liver oil. We have also attempted to concentrate the antirachitic activity of these various butters and of cod-liver oil by the method of alcoholic extraction used by Zucker [1922–23; 1925; 1928] for the preparation of an active concentrate from cod-liver oil.

Experimental.

Methods.

Most of the methods used for the saponification of butter for the preparation of olive oil dilutions of the non-saponifiable residues and for the animal tests have been described in the previous paper [Kon and Booth, 1933, 2]. The short saponification method was used throughout. For feeding, the non-saponifiable residues, Zucker extracts and other substances of which the doses given were too small for administration by 1 ml. calibrated volumetric pipettes were dissolved in ether and mixed with a small quantity of olive oil. After evaporation of the ether *in vacuo*, the remainder of the requisite amount of olive oil was added, the solution was mixed by the aid of a vigorous stream of nitrogen and fed by means of standardised pipettes delivering drops weighing from 19 to 21 mg. The dilution was so adjusted that the requisite dose of non-saponifiable residue or other material was present in from 1 to 4 drops of the solution.

In the distribution of animals for the feeding tests an endeavour was made to divide the litters as evenly as possible among the various substances undergoing test, and as a rule litter-mates were placed in all groups where comparison was especially desirable, such as the comparison between one level of raw butter and its equivalent as non-saponifiable residue. In these, as well as in the previous experiments [Kon and Booth, 1933, 2], not only was the degree of protection

afforded to rats by the various substances measured by the amount of ash in the dry defatted femora and humeri, but photographs were also taken, by the technique described in our previous paper, of sections of radii and ulnae stained with silver nitrate. The large number of rats used in the present tests precludes the publication of these photographs. The results arrived at by a study of these photographs were, in general, in excellent agreement with those based on the ash percentage.

Experiments carried out in the summer of 1933.

Methods. (*a*) *Summer butter from cows at pasture.* The cows went out to grass on April 20th, 1933. Butters from seven bi-weekly churnings were collected between May 12th and June 5th and aliquot parts of these butters, after rendering and filtering, were pooled. This period was very sunny throughout, there being an average of 5·6 hours of sunshine daily, to all of which the cows were exposed. The butter was saponified in the usual way on 3 occasions during the course of the experiment, the non-saponifiable residue being extracted with 4 portions of ether instead of the usual 3.

(*b*) *Butters from cows receiving irradiated yeast*[1]. Two groups of 2 Shorthorn cows each were maintained throughout the winter stall-feeding period 1933 on a ration identical with that fed to the typical herd of Shorthorns [Golding *et al.*, 1932]. Towards the end of this period the ration of one group was supplemented with 50 g. of irradiated yeast daily, while the supplement to the other group was 500 g. daily. In the case of group I, after 14 days of such supplementary feeding butter was made from the cream of the mixed milk of the 2 cows over a 2-day period. The butter was made in the case of group II from a similar sample of cream from the mixed milk of the 2 cows after the yeast had been fed for a period of 3 weeks. These butters were rendered and filtered in the usual way and kept in cold storage until required. They are hereinafter referred to as low-level and high-level yeast butter respectively.

(*c*) *Butter from cows receiving cod-liver oil*[1]. The butter was produced at the same time and under the same conditions as that of the cows receiving irradiated yeast. This group of 2 cows was given, however, daily supplements of 4 to 6 oz. of cod-liver oil for one week and 8 oz. daily per cow for 2 following weeks before the butter was collected.

(*d*) *Irradiated butter.* In preparing this butter we endeavoured to duplicate as far as possible the method of Steenbock and Wirick [1930] with the exception that we used for irradiation a sample of winter butter instead of June butter as used by these authors. The sample of winter butter collected between March 31st and April 4th, 1933, was rendered and filtered in the usual way and stored until required. After melting, 45·36 ml. of the fat were run into a circular bright nickel-plated tray 19 cm. in diameter, thus giving a layer of fat 0·16 cm. thick. The butter was maintained in the fluid state and at a temperature not exceeding 40° by being placed over a hot water-bath during irradiation. This was carried out for $\frac{1}{2}$ hour with a K.B.B. atmospheric type quartz mercury vapour lamp situated at a distance of 45·7 cm. from the melted fat. The lamp had previously been in use for about 200 hours and consumed 2·5 amp. with a potential across the burner of 132 V. After irradiation the butter was kept in cold storage and, when necessary, saponified in the usual way.

(*e*) *Alcoholic extract of winter butter prepared by a method similar to that of Zucker* [1922–23; 1925; 1928]. 250 g. of pooled butter from bi-weekly samples

[1] The experiments dealing with the feeding to cows of irradiated yeast and of cod-liver oil will be reported in detail elsewhere.

collected between March 13th and 27th, 1933, were heated with 150 ml. of absolute ethyl alcohol until completely dissolved. The solution was vigorously shaken and allowed to cool and separate, and the alcoholic layer was then poured off. This extraction was followed by another with 100 ml. of 96 % alcohol and by two further extractions each with 75 ml. of 96 % alcohol. The combined alcoholic extracts were evaporated on the water-bath yielding 17·7 g. of a yellow oil. A part of this oil was saponified in the usual way. The residue from the alcoholic extraction was heated to about 45° and washed in a separating funnel with three successive portions of 100 ml. of hot water. It was then filtered and stored.

In Table I are given the results of the tests.

Table I. *Summer 1933. Prophylactic experiment. Antirachitic effect of various butters, of non-saponifiable residues from these butters and of an alcoholic extract and residue from one of them. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentage are averages of duplicate determinations on groups of 4 rats. In the Tables the dosages of non-saponifiable residues, Zucker extracts, *etc.*, are expressed in the equivalent of the butter from which they were derived.

Positive controls 52·0 % ash
Negative controls (7 rats) 31·2 % ash

Substance tested	Dosage g.	Ash %
Summer butter	0·1	36·1
"	0·2	42·8
"	0·5	49·5
"	1·0	53·5
Non-saponifiable residue of summer butter, equiv.	0·1	36·1
" " "	0·2	38·7
" " "	0·5	44·0
" " "	1·0	49·8
High-level yeast butter	0·01	46·0
"	0·03	50·4
"	0·10	51·1
"	0·25	52·9
Non-saponifiable residue of high-level yeast butter, equiv.	0·01	45·2
" " "	0·03	50·3
" " "	0·10	50·8
" " "	0·25	52·4
Low-level yeast butter	0·05	38·0
"	0·15	49·7
"	0·50	53·5
Non-saponifiable residue of low-level yeast butter, equiv.	0·05	39·1
" " "	0·15	46·0
" " "	0·50	50·6
Cod-liver oil butter	0·10	38·1
"	0·30	48·6
"	1·00	51·8
Non-saponifiable residue of cod-liver oil butter, equiv.	0·10	38·0
" " "	0·30	42·5
" " "	1·00	48·3
Irradiated butter	0·018	49·8
" " "	0·036–0·072	50·4
Non-saponifiable residue of irradiated butter, equiv.	0·018	49·9
" " "	0·036–0·072	50·4
Winter butter	1·00	48·0
Zucker extract of winter butter, equiv.	1·00	35·2
Non-saponifiable residue of Zucker extract of winter butter, equiv.	1·00	35·1
Residue of winter butter after Zucker extraction, equiv.	1·00	46·9

It is apparent from the table that neither the high-level yeast butter nor the irradiated butter has lost its potency as a result of saponification. In the case of the irradiated butter the experiment is unfortunately rather unsatisfactory. Owing to a misjudgment of the degree of protection afforded to the rats receiving the irradiated butter it was decided on the 15th day of the experiment to double the dosage in the case of the animals receiving the higher level of butter and obviously also in the case of the animals receiving the corresponding level of non-saponifiable residue. When the rats were killed it was found that the earlier diagnosis *in vivo* was quite erroneous and that not only the increased higher dosage but also the lower dosage had afforded a high degree of protection. It is therefore possible that, in the case of this butter, some destruction of the antirachitic factor might have taken place upon saponification, but that since the lower dosage was almost sufficient for complete protection, any slight destruction of antirachitic factor would not be apparent.

In the case of the low-level yeast butter, no destruction is evident at the lowest dosage. On the other hand both the 0·15 and 0·5 g. levels of this yeast butter yield a definitely higher ash percentage than their corresponding non-saponifiable residues.

A very similar condition prevails in the case of the cod-liver oil butter. There is no difference between butter and non-saponifiable residue at the 0·05 g. level, but here again, two higher dosages of butter yield higher ash percentages than their corresponding non-saponifiable residues.

The same statement holds true in the case of summer butter.

It will be noticed that the antirachitic activity of summer butter withstands saponification to a greater extent than that of autumn and winter butters. Our observations on butters collected in summer 1933 are in complete agreement with a preliminary test carried out on butter collected in the summer period 1932 previously recorded by us [Kon and Booth, 1933, 1].

With regard to the Zucker extract of winter butter it is quite evident that, as far as this experiment goes, it would confirm Zucker and Barnett's opinion [1922–23], quoted in our previous paper [Kon and Booth, 1933, 2], that the antirachitic activity of butter cannot be concentrated by their method. It is obvious that the loss is not due primarily to destruction of the antirachitic factor by saponification, as practically the whole of the activity was left in the residue, and the saponification did not in this case further diminish the already slight activity of the alcoholic extract.

Experiments carried out in autumn 1933, first series.

In this period we repeated our summer experiments with irradiated butter using dosages of 5 and 10 mg. These dosages were intended to afford an incomplete protection in order to obtain an unequivocal result as to whether or not destruction occurred in this butter on saponification. We also prepared and tested in this period Zucker extracts of high-level yeast butter, irradiated butter, winter butter and cod-liver oil. It was obvious that before reaching any definite conclusions with regard to the inapplicability of Zucker's method to butter, we had to make sure that we could obtain by this method, and under our laboratory conditions, an active concentrate from cod-liver oil. As an additional check on our previous findings as to the effect of saponification upon the antirachitic potency of various butters we again saponified a sample of winter butter.

Methods. (*a*) *Irradiated butter.* The same sample was used as in the previous summer experiment. This had been kept in the ice-chest throughout the intervening period. The methods of saponification and of feeding the butter and its

non-saponifiable residue were exactly as previously described, but four ethereal extractions of the non-saponifiable residue were made from the saponification mixture.

(*b*) *Preparation of the Zucker extracts.* For the preparation of Zucker extracts we used the same high-level yeast butter and irradiated butter as in our summer experiment. The cod-liver oil was from the bulk given us by Dr Coward for use in our previously reported experiments [Kon and Booth, 1933, 2], while the winter butter was from a pooled sample collected bi-weekly between February 10th and March 14th, 1933. The Zucker extracts of all these substances were made in exactly the same way. 20 g. of the substance to be extracted (200 g. in the case of winter butter) were mixed with 25 ml. of absolute alcohol (250 ml. in the case of winter butter) and were shaken on a mechanical shaker for 1½ hours. The butter-alcohol mixtures were, before shaking, heated sufficiently to melt the butter-fat. In all, four extractions were made, the same amount of alcohol being used for the second extraction as for the first, while in the last two extractions the amount of alcohol used was halved. After each of the first three extractions the oils were allowed to settle, and the alcoholic layer was siphoned off; after the last extraction the mixtures were centrifuged before the alcoholic layer was siphoned off. In each case the bulked extracts were concentrated on the water-bath, while the residues were washed four times with hot distilled water. The following yields were obtained:

Cod-liver oil	2 g. extract from 20 g. original oil.
Irradiated butter	5·08 g. extract from 20 g. original butter-fat.
High-level yeast butter	5·69 g. extract from 20 g. original butter-fat.
Winter butter	52·15 g. extract from 200 g. original butter-fat.

No special effort was made to recover the residues quite quantitatively, as the amounts used were far in excess of the requirements for the feeding tests, but for the purposes of dilution and feeding it was always assumed that the weight of the residue was equal to the weight of the original substance less the weight of the extract. It will be noticed that this method of extraction, which follows almost exactly that recommended by Zucker [1925; 1928], gives a much greater yield of extract from butter than the modification used in our summer experiment.

The methods of saponification and feeding of the Zucker extracts and residues and of their non-saponifiable residues were as previously described. The same applies to the saponification and feeding of winter butter; in all cases the soaps after saponification were extracted with four successive quantities of ether.

In Table II are given the results of these tests.

It can be seen that neither of the two levels of irradiated butter fed this time was sufficient to bring about complete protection. While the non-saponifiable residue of the lower level gives a slightly lower percentage of ash than the butter itself the reverse is true in the case of the higher level, and as these differences are well within the experimental error it is obvious that the antirachitic factor of irradiated butter can be quantitatively recovered in the non-saponifiable residue of the latter.

The saponification of winter butter has again resulted in a very incomplete recovery of its antirachitic activity in the non-saponifiable residue.

The results with the Zucker extracts have been rather unexpected. It is obvious from our figures that our inability to extract the vitamin D potency by this method is not restricted to winter butter alone, or even to butters of an enhanced antirachitic potency completely recoverable after saponification, but extends to cod-liver oil. It will be noticed that, while in the case of the latter the

Table II. *Autumn 1933. Prophylactic experiment, first series. Antirachitic effect of Zucker extracts and residues from cod-liver oil and various butters before and after saponification; antirachitic effect of the original butters and cod-liver oil and of the non-saponifiable residues of two of the butters. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.
Negative controls 34·9 % ash

Substance tested	Dosage g.	Ash %
High-level yeast butter	0·010	44·8
Zucker residue of high-level yeast butter, equiv.	0·010	33·7
Zucker extract of high-level yeast butter, equiv.	0·010	35·7
Non-saponifiable residue of Zucker extract of high-level yeast butter, equiv.	0·010	35·9
Cod-liver oil	0·0008	48·4
Zucker residue of cod-liver oil, equiv.	0·0008	44·7
Zucker extract of cod-liver oil, equiv.	0·0008	32·9
Non-saponifiable residue of Zucker extract of cod-liver oil, equiv. ...	0·0008	33·9
Irradiated butter	0·005	44·8
"	0·010	46·9
Non-saponifiable residue of irradiated butter, equiv.	0·005	43·7
" "	0·010	47·6
Zucker residue of irradiated butter, equiv.	0·010	35·9
Zucker extract of irradiated butter, equiv.	0·010	37·9
Non-saponifiable residue of Zucker extract of irradiated butter, equiv.	0·010	38·0
Winter butter	0·700	47·3
Non-saponifiable residue of winter butter, equiv.	0·700	35·4
Zucker residue of winter butter, equiv.	0·700	37·2
Non-saponifiable residue of Zucker residue of winter butter, equiv. ...	0·700	33·6
Zucker extract of winter butter. equiv.	0·700	36·4
Non-saponifiable residue of Zucker extract of winter butter, equiv. ...	0·700	33·1

residue after extraction seems to have retained a large part of its antirachitic potency, in the case of all the butters both the extract and the residue are only very slightly active. But even here the extract and residue of winter butter have suffered further loss on saponification.

Shipley *et al.* [1924] report that alcoholic extracts of butter-fat may contain appreciable amounts of antirachitic potency while the alcohol-insoluble residue was found by them to be inactive.

Experiments carried out in autumn 1933, second series.

Autumn butter collected in this period was tested for its antirachitic potency before and after saponification. The results of these tests were again in complete agreement with our previous experience with autumn and winter butters. At the same time, other experiments were carried out. These will be reported in the following paper.

Methods. The butter used in this experiment was collected between October 16th and November 16th, 1933. Five saponifications were carried out, the last four on the previous week's churnings, while in the first one a 3 days' sample was used. In order to ensure as perfect a recovery as possible the non-saponifiable residue was extracted on these occasions 5 times with ether.

The autumnal rains which followed the very dry and hot summer of 1933 had produced an exceptionally good crop of grass for this time of the year, and the butters, especially the first 2 or 3 samples, were therefore much more highly coloured than is usual under our conditions at this season. In fact the yellow

pigmentation and the vitamin A content of these butters were higher than those of the summer butter tested in the first series of experiments reported in this paper. On the other hand the actual sunshine to which the cows were exposed was in this case only 3·16 hours daily. The intensity of solar radiation at this time of the year was also obviously less than in summer. Observations on the colour and vitamin A activity of these butters will be reported in detail elsewhere. It might be mentioned here, incidentally, that the depth of yellow coloration and/or of vitamin A activity of a butter cannot be used as a reliable guide to its vitamin D activity.

In Table III are given the results of the tests.

Table III. *Autumn 1933. Prophylactic experiment, second series. Antirachitic effect of autumn butter and of its non-saponifiable residue. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.

Positive controls (2 drops of c.l.o. daily) 52·3 % ash
Negative controls 33·2 % ash

Substances tested	Dosage g.	Ash %
Autumn butter	0·25	40·8
,,	0·50	47·1
,,	1·00	52·9
Non-saponifiable residue of autumn butter, equiv.	0·25	34·6
,, ,,	0·50	39·1
,, ,,	1·00	40·3

This autumn butter is more potent than that collected at the same time during the previous year; on the other hand it is less potent than the sample of butter collected and tested in summer 1933. The loss of potency after saponification is quite marked and, as the non-saponifiable matter had been in this case so thoroughly extracted, this loss of potency in the course of saponification must be real and cannot be due to faulty extraction.

Experiments carried out in autumn 1933, third series.

The experiments carried out in this series were chiefly of a routine nature and formed part of our regular estimation of the antirachitic factor of butter by the curative line test technique. This latter has been described in our previous paper [Kon and Booth, 1933, 2]. The autumn butter was fed at three levels: 0·25, 0·5 and 1·0 g. daily before and after saponification.

The two lower levels of butter failed to give any definite signs of healing while 4 out of 5 rats receiving 1·0 g. of butter showed such signs varying in intensity from an indication of a line to a medium broken line. The lower potency of the non-saponifiable residue is shown by the fact that in only 2 out of 5 rats receiving the highest level are specks of calcification to be found.

Discussion.

Before devoting our attention to what we believe to be the positive results of our work, we would like to discuss briefly a negative finding, *i.e.* our inability to obtain an active extract from cod-liver oil by the method employed by Zucker [1922–23; 1925; 1928]. When in the summer of 1933 we prepared the Zucker extract

from butter and found it inactive we thought that this finding very definitely lent support to our contention that a chemical difference exists between the antirachitic agent of butter and other antirachitic agents. Obviously the validity of this finding stood or fell with our ability to obtain by the same method an active extract from cod-liver oil. Our failure to obtain such an extract is definitely not due to a destruction in the course of extraction, as the residue after extraction was found to contain the major part of the original activity. That highly active concentrates can be, and are, prepared from cod-liver oil by the method of Zucker we do not doubt. Such extracts are produced under Zucker's patents [Zucker, 1925; 1928] on a large scale in the United States and are, as Zucker [1933, 1, 2] reports, regularly used for enhancing the antirachitic potency of bread and milk.

We do not know what grades of cod-liver oil are used in this process. The cod-liver oil unsuccessfully used by us was one of very high vitamin D content (250–300 International units per g.) and was a high grade medicinal oil kindly given us by Dr Coward. It is conceivable that the acidity of the oil may play a vital part in the process of extraction, and that vitamin D could be extracted from oils of high acidity but not from neutral oils. This is merely an assumption, but the fact remains that our results in this respect are of no use in elucidating the difference between the antirachitic factor of butter and that from other sources.

In our first paper [Kon and Booth, 1933, 2] we reported observations on the effect of saponification on butters collected in autumn and winter. We observed that only a small fraction of the original antirachitic activity of these butters could be recovered in the non-saponifiable residues. At that time we abstained from making any suggestions regarding the possible abnormal behaviour of the antirachitic activity of butters in general, especially as we had been led by preliminary experiments [Kon and Booth, 1933, 1] to believe that butters may differ in this respect. Since then we have examined a variety of butters, and we have found that while the activity of some of them is markedly reduced by saponification, the antirachitic potency of others will withstand this treatment just as well as those of cod-liver oil and irradiated ergosterol.

When the butters examined by us are placed in an ascending series according to the degree of recovery in the non-saponifiable residue of their original antirachitic activity, it becomes at once evident that these butters are also graded in another respect—namely that they have been simultaneously arranged in ascending order of original antirachitic potency. In other words, the greater the potency of the butter, the more complete in our experience is the recovery of this potency after saponification.

The loss of potency on saponification is never complete, a loss of about 80 % of the original potency seems to be the maximum we have experienced. Such is the case with the least potent of the butters collected in autumn and winter. Any increase of potency above this practically basal level is accompanied by a corresponding, and, apparently, quantitive increase in the activity recoverable after saponification. We are therefore led to believe that at least two factors antirachitic for the rat are present in the butters which we have so far examined. One of them is clearly destroyed or diverted into a fraction other than the non-saponifiable residue during the course of saponification. The other behaves in an orthodox way. Whether this latter factor is to be identified with the vitamin D of cod-liver oil, whether it is the same as irradiated ergosterol, or whether, possibly, it differs from those two recognised factors, we are at present obviously not in a position to say. We only know that it shares with those two factors their resistance to saponification. It has been so far our experience that, by whichever method we

attempted to increase the antirachitic activity of the butter, the added factor behaved in a normal way.

It would therefore appear that while the cow possesses the ability of secreting into her milk not only vitamin D but also another substance or property antirachitically active for the rat, the yield of this substance cannot be influenced either by changes in the natural conditions such as changes from winter to summer types of feeding, or by massive feeding of highly potent carriers of vitamin D activity, or, lastly by direct irradiation of the butter. The cow thus appears to be able to produce a novel antirachitic agent, but definitely does not rely for its production upon any exogenous source of vitamin D *sensu stricto*, whatever this term implies. A physiological conversion of added known forms of vitamin D into the novel form found in butter has not, in our experience, been found to occur.

As far as we can judge, this factor would seem constant in all the butters and always present in the same amount in a given bulk of butter. Thus, for example, it might constitute 75 % of the antirachitic activity of a butter of which 1 g. daily is required to give almost complete protection. When on the other hand the activity of such a butter is raised nearly a hundred times and 10 mg. of this butter nearly suffice to afford complete protection, the orthodox factor only is increased and the rôle played by the unresistant factor becomes so small that its loss on saponification is not noticeable.

Summary.

1. The following butters have been tested for their antirachitic potency both before and after saponification: (*a*) butter from cows on summer pasture, (*b*) butters from cows having received two different levels of irradiated yeast, (*c*) butter from cows having received cod-liver oil, (*d*) irradiated winter butter. It was found that the antirachitic value of high-level yeast butter and of irradiated butter could be recovered practically completely in the non-saponifiable residue, while the remaining butters suffered a more or less marked loss in the course of saponification. These butters, however, including summer butter, were definitely more resistant to saponification than the autumn and winter butters reported on in the previous paper of this series.

2. In confirmation of previous work it was found that a large part (over 75 %) of the potency of autumn and winter butters was either destroyed or diverted to another fraction on preparation of the non-saponifiable residues.

3. The greater the initial antirachitic potency of the butter, the greater is the proportion of the potency recoverable in the non-saponifiable residue.

4. It is suggested that at least two factors antirachitic for the rat are present in butter; a factor which is not recoverable in the non-saponifiable residue, and the usual vitamin D (whatever this term may imply), which is resistant to saponification. By exposure to summer conditions or by the feeding to cows of highly antirachitic substances such as irradiated yeast or cod-liver oil the concentration of the stable factor but not of the labile factor of butter can be markedly increased. Direct irradiation of the butter also leads to the formation of the stable factor only.

5. Attempts to achieve a chemical separation of the labile and stable antirachitic factors of butter by submitting butters of varying potency to extraction with alcohol, following the method of Zucker, failed in so far as we were unable, contrary to Zucker's findings, to extract by this method the antirachitic potency of a high grade cod-liver oil, or, in agreement with Zucker, to extract that of butter.

Our best thanks are due to Capt. J. Golding and Prof. H. D. Kay for helpful criticism, to Miss D. V. Dearden for the churning of butters and to Mrs S. K. Kon for much help in the preparation of bones for analysis.

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XVIII. THE VITAMIN D ACTIVITY OF BUTTER. III. AN ATTEMPT TO ELUCIDATE THE NATURE OF THE LABILE FACTOR IN BUTTER ANTIRACHITIC FOR THE RAT. THE ANTIRACHITIC POTENCY OF LARD, OLIVE OIL, EGG OIL AND THE FATTY ACIDS OF BUTTERS AND LARD.

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(*Received January 1st, 1934.*)

In the previous paper of this series [Kon and Booth, 1934] we have summarised the evidence which points to the existence in butter of two factors antirachitic for the rat, the differentiation between the two factors being based upon the inability to recover one of them in the non-saponifiable residue of butter.

The present paper deals with a description of our efforts to elucidate the nature of the labile factor.

Our experiments were carried out in three main directions.

1. Firstly, we thought it advisable to investigate the effect of saponification on the antirachitic potency of eggs. With the exception of fish-liver oils and of some oils of marine animals, egg oil is probably the most concentrated natural source of vitamin D activity. Excluding such additions to the diet as cod-liver oil and fish meal it would seem that the exogenous sources of the antirachitic factor, or factors, available to the laying hen kept on open range are very similar to those placed at the disposal of the cow. Exposure to sunshine is common to both, as is also the consumption of grass, though in the latter case obviously in different relative quantities. We know of no marked difference in the qualitative or quantitative sense between the vitamin D activity, if any, of the other basal vegetable constituents of the diet of either the hen or the cow.

We therefore studied the effect of saponification on the antirachitic activity of eggs obtained from hens kept on open range and receiving no addition of either fish meal or cod-liver oil to their diet.

2. Secondly, we examined the fatty acid fraction of butters for antirachitic action.

Some ten years ago, Zucker and Barnett [1922–23], in trying to explain their failure to concentrate the vitamin D activity of butter in a manner successfully used by them in the preparation of an active concentrate from cod-liver oil, advanced the hypothesis that the antirachitic activity of butter as measured on the rat might be largely, if not solely, due to the action of the fatty acids of butter combining with the dietary calcium and causing the excretion of increased amounts of calcium from the gut in the form of calcium soaps. While we do not necessarily agree with Zucker and Barnett's explanation of the mechanism of fat action, we were nevertheless impressed, as they were, by the possibility of the saponifiable fraction of butter playing a rôle in the total antirachitic activity

of the latter. We have therefore planned and carried out experiments which, in our opinion, might help in elucidating the problem.

Zucker and Barnett's suggestion, as far as we understand it, was made in an attempt to reconcile the success of their method of concentrating the antirachitic factor of cod-liver oil with the failure of this method as applied to butter and other substances. It was not based on experimental evidence derived from the feeding to rats of the fatty acids of either butter or of the vegetable fats mentioned in their paper. We [Kon and Booth, 1934] have not only failed to extract by the above method the antirachitic activity of butter, but were equally unsuccessful in the case of cod-liver oil. On this basis, therefore, we see no *a priori* reason for ascribing the antirachitic activity of butter, partly or wholly, to its fatty acid moiety. Nevertheless, the peculiarities of the etiology of experimental rickets in rats make it imperative to investigate experimentally the possible rôle of the fatty acid fraction of butter. The rachitic condition in the rat cannot be brought about by a deficiency of vitamin D alone. A rachitogenic diet must also be grossly abnormal in the proportion of its mineral constituents in order to produce a good degree of rickets. It is well known [Karelitz and Shohl, 1927; Brown *et al.*, 1932; Shohl *et al.*, 1932; Key and Morgan, 1932] that the mere addition of phosphorus to a rachitogenic diet, or the withdrawal of a part of the calcium, produces the same effect as if an antirachitic substance had been added to the otherwise unchanged diet. It is, therefore, evident that any substance which would alter the calcium and phosphorus metabolism of the rachitic rat, either by increasing the excretion of calcium or by facilitating the uptake of phosphate from the gut, will behave, in bringing about a prevention or cure of the rachitic condition, in a manner indistinguishable from that of a true antirachitic agent.

The smaller the original potency of a butter the larger is the amount which it is necessary to feed to ascertain its antirachitic activity. It should be remembered that, as the fatty acids of butter weigh almost as much as butter itself, the amounts of fatty acids which it is necessary to administer to the rat when testing butters of low or moderate activity are quite considerable. There is ample evidence which has been lately summarised by Boyd *et al.* [1932] that fatty acids play a very definite rôle in the metabolism of calcium and phosphorus in the rat.

Under the second heading also must come our experiments in which we fed to rats the non-saponifiable residue simultaneously with the fatty acid fraction of butter. If that part of the activity of butter which is not recoverable after saponification is due to the antirachitic action of the fat, then such a "reconstituted" butter should regain its original activity.

We found it impossible to feed fatty acids separately from the diet by pipette as we feed butters. The higher melting-point formed one obstacle. A more serious one was the refusal of the rats to consume the fatty acids when given by pipette. These had therefore to be fed incorporated in the diet and as a check we thought it desirable to feed butter likewise incorporated in the diet.

3. Lastly, we fed to rats a vegetable fat, olive oil, and an animal fat, lard, in quantities comparable with those administered in the case of butters of low or moderate antirachitic activity.

Olive oil is generally recommended for use and is used as a diluent for cod-liver oil and a solvent for irradiated ergosterol. In small quantities high grade olive oil which has not been exposed to sunlight is antirachitically quite inert. The effects, if any, of large quantities of this fat would therefore, in our opinion, throw a valuable light on the fatty acid hypothesis of the antirachitic activity of butter.

The use of lard as a constituent of a rachitogenic diet composed mainly of purified foodstuffs has been suggested by Osborne and Mendel [1923] and Smith and Mendel [1930]. These authors advise the use of diets containing lactalbumin or edestin, starch, a phosphorus-free salt mixture and from 0 to 24 % lard. For this reason we chose lard as a representative animal fat which might be considered to be antirachitically inert.

Experimental.

Methods.

1. *Egg oil.* Fresh eggs were obtained at the end of June 1933 from Light Sussex hens on open range and receiving a grain mixture containing meat meal but no cod-liver oil or fish meal. Eggs were also collected at the end of October 1933 from the same hens receiving the same diet.

The yolks of the eggs were carefully separated from the whites, and after adding an equal volume of 8 % NaCl [Jukes and Kay, 1932] they were extracted three times with ether, the third extract being almost colourless. The extract was washed twice with water, dried over anhydrous Na_2SO_4 and the ether evaporated after filtering. For feeding, the egg oil was diluted with olive oil in the usual way. After saponification of 10 g. of egg oil the mixture of soaps was extracted seven times with ether using one portion of 75 ml. and six portions of 50 ml. This number of extractions was necessary in order to remove the bulk of the colouring matter (xanthophyll). It is very probable that a smaller number of extractions would have effected a quantitative recovery of the antirachitic factor. The non-saponifiable residue was dissolved in olive oil for feeding as previously described.

2. *Fatty acids.* All the fatty acids discussed below were prepared by the following method. After removal of the non-saponifiable residue from saponified butter or lard the soaps were acidified with sulphuric acid and the fatty acids extracted three times with ether. The ethereal solution was washed twice with water and dried over anhydrous Na_2SO_4. The ether was then removed, firstly on the water-bath and then under diminished pressure. This method of recovery is not quite quantitative, losses occurring in the lower volatile and water-soluble fatty acids. For feeding, the fatty acids were incorporated in diet 2965 of Steenbock by dissolving them in ether, mixing the solution with the diet and subsequently allowing the solvent to evaporate. The fatty acids derived from the following butters and lard were prepared and fed[1]: summer butter; irradiated butter; autumn butter; high-level yeast butter; winter butter; home-rendered lard. Records of food intake of the several groups of rats receiving these fatty acids were kept. As in the case of all other substances tested, each fatty acid and level of fatty acid was fed to groups of 4 rats kept in one cage. The food records give therefore the average amount of fatty acid consumed by any rat in the group. It will be remembered [Kon and Booth, 1933; 1934] that all substances fed separately from the diet were administered on weekdays only, while the substances incorporated in the diet were given seven times a week. Therefore to give a true impression of the antirachitic activity, if any, of the fatty acids we give in the following table average weekly consumptions. The fatty acids were usually fed in the same experiments as those involving the feeding of the corresponding original fats and their non-saponifiable residues. Whenever several samples of butter were saponified in the course of one feeding test, fresh

[1] The corresponding butters were discussed in our previous paper [Kon and Booth, 1934].

samples of the corresponding fatty acid-containing diets were prepared on the same days as those on which the changes were made in the feeding of the fats and non-saponifiable residues.

When fatty acids were fed to rats simultaneously with the administration of the non-saponifiable residues, the fatty acids were fed exactly as described above, and the non-saponifiable residues were administered six times a week in the usual way.

For feeding in the diet the butter-fat was melted and poured over the requisite amount of the 2965 rachitogenic diet and was then well kneaded in by hand until a perfectly homogeneous material was obtained. The bulk of this was stored in a cold place. These diets were fed and food intake was recorded in the same way as in the case of fatty acids.

3. (*a*) *Olive oil.* The olive oil was fed by pipette separately from the diet in an exactly similar manner to the butters. It was a high grade first pressing Italian oil which had been used by us with complete satisfaction as a diluent for cod-liver oil and the International Standard of Vitamin D, and as a solvent for the various non-saponifiable residues and other substances requiring dilution.

(*b*) *Lard.* Three samples of lard were tested, two of them were purchased from a local grocer and were both of American origin. It is quite possible that they may have been both from the same consignment, but we prefer to consider these lards as separate specimens. The second sample was from a bulk labelled: "White Rose Pure Lard, Dold Quality, Buffalo, U.S.A."

The third sample was a lard rendered by us from the leaf fat of 4 pigs slaughtered at bacon weight at the end of September 1933 when 7 months old. For the last 4 months of life they had been kept indoors without access to sunlight and grass and had been maintained on the following diet:

Barley meal	48·0 %	Middlings (Weatings)	35·0 %
Soya-bean meal	6·0	Meat meal	4·0
Dried whole milk	5·0	Ground limestone	1·75
		Sodium chloride	0·25 %

It is reasonable to suppose that the vitamin D reserves, if any, of these animals were of a low order. For rendering, the chopped fat was heated in a pail immersed in boiling water and the fat filtered through cheese-cloth, allowed to settle and set, remelted and filtered through a fluted filter.

The first sample of lard was fed only as such. The second and third samples were fed both before and after saponification. The non-saponifiable residues were extracted four times with ether. The lards were given by pipette separately from the diet. One level of the first sample was fed mixed in the diet.

It will be seen from Table I that there is no difference between the anti-rachitic effects of doses of summer egg oil and those of corresponding levels of the non-saponifiable residues of the same oils. On the other hand the highest of the three levels tested did not afford more than a slight degree of protection. A reference to the previous paper of this series [Kon and Booth, 1934] will show that, although in the case of summer butter and of cod-liver oil butter there was no difference between the antirachitic effects of the butter and of its non-saponifiable residue at the lowest level of intake, which gave an ash percentage similar to that produced by the highest level of egg oil, definite differences could be noted at the higher levels. In the light of our experience with summer egg oil the levels of autumn egg oil were better selected, but unfortunately an error was made in the dilution of the non-saponifiable residue which was made up to twice

Table I. *Summer and autumn 1933. Prophylactic experiments. Antirachitic effect of various egg oils, lards, butters and their non-*

Table I. *Summer and autumn 1933. Prophylactic experiments. Antirachitic effect of various egg oils, lards, butters and their non-saponifiable residues; olive oil; fatty acids of lard and butters; fatty acids and non-saponifiable residue of butter fed together; butter incorporated in the diet. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.
The dosages of non-saponifiable residues are expressed in equivalents of the butter or lard from which they were derived.

Substance tested	Dosage g.	Ash %
Summer egg oil	0·001	30·5
Summer egg oil	0·003	34·1
Summer egg oil	0·006	38·9
Non-saponifiable residue of summer egg oil, equiv. ...	0·001	30·1
Non-saponifiable residue of summer egg oil, equiv. ...	0·003	34·0
Non-saponifiable residue of summer egg oil, equiv. ...	0·006	38·2
Autumn egg oil	0·0115	41·2
Autumn egg oil	0·0345	51·5
Non-saponifiable residue of autumn egg oil, equiv. ...	0·0230	42·2
Non-saponifiable residue of autumn egg oil, equiv. ...	0·0690	52·0
Olive oil	1·0	37·4
Negative controls for olive oil	—	30·1
American lard, 1st sample	0·25	43·9
American lard, 1st sample	0·50	51·2
American lard, 1st sample	1·00	51·5
American lard, 1st sample incorporated in the diet, approximately	1·50	49·2
American lard, 2nd sample	0·5	48·5
American lard, 2nd sample	1·0	51·3
Non-saponifiable residue of American lard, 2nd sample, equiv.	0·5	41·5
Non-saponifiable residue of American lard, 2nd sample, equiv.	1·0	45·4
Home-rendered lard	1·0	48·0
Non-saponifiable residue of home rendered lard, equiv.	1·0	34·5
Negative controls for American lard, 2nd sample and for home-rendered lard	—	34·9

Substance tested	% in diet	Av. weekly intake of substance per rat g.	Ash %
Summer butter	Fed separately	3·0	49·5
Summer butter fatty acids	6·25	3·0	37·6
Negative controls at this time	—	—	32·0
Irradiated butter	Fed separately	0·06	46·9
Irradiated butter fatty acids	0·20	0·15	30·9
Winter butter	Fed separately	4·20	47·3
Winter butter fatty acids	7·00	3·23	35·8
Negative controls for irradiated butter and winter butter	—	—	34·9
Autumn butter	Fed separately	3·00	47·1
Non-saponifiable residue of autumn butter	Fed separately, equiv.	3·00	39·1
Autumn butter fatty acids	6·25	3·26	36·7
Autumn butter fatty acids + non-saponifiable residue	6·25	3·37	41·2
Autumn butter in diet	6·25	3·78	43·3
Autumn butter	Fed separately	6·00	52·9
Non-saponifiable residue of autumn butter	Fed separately, equiv.	6·00	40·3
Autumn butter fatty acids	12·50	6·16	40·3
Autumn butter fatty acids + N.S.R.	12·50	5·75	50·2
Autumn butter in diet	12·50	6·80	48·5
Negative controls for autumn butter	—	—	33·2
High-level yeast butter	Fed separately	0·06	46·0
High-level yeast butter fatty acids	1·25	0·75	43·1
Negative controls for high level yeast butter	—	—	32·0
Home-rendered lard fatty acids	12·50	5·60	39·7
Negative controls for these fatty acids	—	—	33·2

the concentration of the original oil. The results might be interpreted as indicating a certain loss of the antirachitic potency during the course of saponification. We would prefer for the time, however, to consider these figures as inconclusive. It should be mentioned here that Hess and Weinstock [1924] found that the non-saponifiable residue of egg-yolk was antirachitically active and obtained a preparation about 90 times as concentrated as the original yolk. We are planning to investigate the effect of saponification on eggs from the same source as those obtained and tested by us in summer and autumn, but collected in February when their antirachitic potency should be at its lowest.

The feeding of the olive oil at a 1 g. level had a definite effect upon the severity of the rachitic condition. It will be noticed that the bones of rats receiving this quantity of olive oil contained 7 % more ash than those of the negative controls.

The results of feeding the first sample of the American lard were quite startling. The antirachitic effect of this lard was, if anything, better than that of the summer butter fed at the same time. (The figures for this butter are given in the previous paper [Kon and Booth, 1934].) No records of food consumption were kept in the case of lard incorporated in the diet, but an average consumption of 8 g. per day would imply the ingestion of 1·5 g. of lard. It would appear that lard incorporated in the diet is less antirachitically effective than when administered separately from it.

The second sample of American lard was also, comparatively speaking, very potent and gave results similar to those produced by the first sample. The non-saponifiable residue gave definitely lower values than the lard itself, and the degree of loss was very similar to that which occurred in the case of summer butter.

The home-rendered lard was less potent than the two American lards. Very marked loss took place here after saponification, the rats receiving the non-saponifiable residue being indistinguishable from negative controls.

Drummond *et al.* [1920], in a paper written before vitamins A and D were differentiated, stated that the addition of lard obtained from pigs receiving an ample supply of the fat-soluble vitamin definitely increased the growth and improved the condition of rats kept on a diet deficient in this factor, while lard from a pig without such supply of vitamin failed to do so. Quite recently (since this work was started) Read and Bailey [1933] have stated that some lards may contain an appreciable amount of vitamin D, while others contain an inappreciable amount of this factor.

On reviewing the antirachitic effect of fatty acids it is apparent that their action, though slight, is unmistakable. With the sole exception of those of high-level yeast butter the fatty acids, even at a level of 1 g. daily, do not increase the ash beyond 40 %. The case of the high-level yeast butter fatty acids requires some explanation. These fatty acids fed at a level of approximately 0·12 g. daily produced an ash percentage of 43·1, quite definitely higher than that given at the same time by the negative controls. On the other hand the degree of protection afforded by this amount of fatty acid is lower than that given by one-twelfth this amount of the original butter. This was the only fatty acid of this series which was separated from the non-saponifiable residue by only three ether extractions. If only 5 % of the non-saponifiable residue were left in the fatty acids, which under these circumstances is quite possible, the activity of this sample of fatty acids would be accounted for.

In the case of the remaining fatty acids which had all been separated from their non-saponifiable residues by four or, in the case of autumn butter, five extrac-

tions, no activity ascribable to the non-saponifiable fraction would seem to be left in the acids. The activity of the acids does not seem to bear any relation to the original total activity of the fat but appears to be proportional to the amount of fatty acid consumed. Thus for example the fatty acids of irradiated butter, though fed at almost three times the level of the original irradiated butter, gave an ash percentage actually lower than that given by the negative controls for this group of experiments, though the irradiated butter fed at the lower level afforded almost complete protection.

The combination of fatty acids and non-saponifiable residue of autumn butter gave, at the higher level of 1 g., only a slightly lower value than that produced by 1 g. of untreated butter. On the other hand the combination of fatty acid and non-saponifiable residue proved definitely less effective at the lower level of 0·5 g. than the corresponding amount of untreated butter.

When the butter was incorporated in the diet instead of being fed separately from it, the ash percentages were definitely lower in the former than in the latter case, although on the basis of food consumption slightly more butter was consumed by the animals which received it as an admixture to the diet. As already stated we noticed this phenomenon also in the case of lard.

Discussion.

In order to appraise the significance of the experimental results described in this paper the following points should, in our opinion, be taken into consideration.

Firstly, with regard to its antirachitic properties lard behaves in a way very similar to that of butter. Different samples of lard display activities ranging from that of winter to that of summer butter, the antirachitic potency being lost on saponification in a way closely resembling that found in the case of butter, the loss being less marked in the more potent sample.

Secondly, when fed at 0·5 and 1·0 g. levels all fatty acids seem to have had a definitely measurable antirachitic effect. It seems extremely doubtful whether this effect can be attributed to incomplete removal of the stable vitamin D, as the amount of total activity which would have to remain in the fatty acids in order to produce such an effect would be far too great in the case of butters of originally low activity. Even on the unlikely assumption that, after 4 or 5 extractions with ether, 5 % of the orthodox activity had been left in the fatty acids, the amount so left in the case of autumn butter or of home-rendered lard would be quite insufficient to raise the ash percentage so definitely above the level of the negative controls. The completely negative results in the case of the fatty acids of irradiated butter, which were fed at a level relatively (on the basis of original potency) more than double that of the fatty acids of the butters and lard of low activity, also point in the same direction.

Thirdly, when butter is incorporated in the diet, its antirachitic effect is definitely lower than that of an equivalent amount of butter fed separately. The same phenomenon can also be noticed in the case of lard. It should be emphasised here that this difference in potency according to the method of feeding cannot in itself explain or account for the very marked drop in potency caused by saponification. A comparison of the ash percentages produced by 0·5 and 1·0 g. of autumn butter fed in the diet with those for corresponding levels of the same butter after saponification given in Table I bears out this statement. As already mentioned we have been technically unable to feed the fatty acids separately from the diet. Should they behave in a way similar to that of the original butter, then their antirachitic effect would be more marked when fed separately.

Fourthly, a combination of the fatty acids and non-saponifiable residue of autumn butter elicits an antirachitic effect superior to that produced by either component fed separately. As the fatty acids were fed in the diet we think that the antirachitic effect of such a "reconstituted" butter should be compared with that of corresponding amounts of the original butter also incorporated in the diet. When this is done it will be found that the difference in antirachitic effect is so slight as to be well within the limits of experimental error. In other words, it would seem that the total activity of butter (of this particular sample of autumn butter in any case) can be recovered quantitatively after saponification when the fatty acid moiety of the butter is recombined with the non-saponifiable fraction.

We have proved to our entire satisfaction in the two previous papers of this series [Kon and Booth, 1933; 1934] that the loss of potency following saponification evident in certain butters cannot be attributed to incomplete extraction of the non-saponifiable residue. Bearing this in mind and adding the evidence just discussed we can arrive at the following alternative suggestions as to the nature of the labile antirachitic factor of butter.

A. The first possibility would be to accept the existence in butter and in lard of a novel antirachitic factor unstable to saponification, and in general more labile than the usual vitamin D. This factor would appear to lose a part of its potency immediately after the butter or lard is mixed with the 2965 rachitogenic diet even when it is kept thereafter at a fairly low temperature; conditions under which irradiated ergosterol and cod-liver oil are stable for longer periods. The existence of such a factor seems quite conceivable were it not for the fact that it is difficult to account on this basis for the slight but unmistakable residual activity of the fatty acids. Also, if the existence of the novel factor should be the true explanation, it is very difficult to understand why a combination of fatty acids and non-saponifiable residue should have virtually the same antirachitic activity as corresponding levels of butter incorporated in the rachitogenic diet.

B. It might also be suggested that the antirachitic activity of butter was the resultant of two factors, namely, the activity of vitamin D *sensu stricto*, whatever this term implies, and of the antirachitic action of fat. In other words it might be suggested that the rat gives, because of its physiological peculiarities, an enlarged and distorted picture of the "true" antirachitic activity of butter, reflecting not only a real antirachitic factor pharmacologically active in different species, but also a non-specific stimulus antirachitic only in the narrow "rat" sense of the word. There is much in our experimental evidence in favour of such a view. The residual activity of the fatty acids, remarkably constant in the case of butters and lard and independent of the activity of the parent substance, and the possibility of reconstructing the original activity of butter by combining fatty acids and non-saponifiable residue speak strongly in favour of such an hypothesis. This explanation has also in its favour the argument of simplicity, as it avoids the necessity of adding another general antirachitic factor to the two already existing. It explains without difficulty why lard should behave in a similar way to butter and also accounts for the slight activities of various vegetable oils reported in the literature and found by us in the case of olive oil.

If also that part of the antirachitic activity of butter which cannot be recovered after saponification is not due to the presence of a specific antirachitic factor but to the action of fat, it is obvious that any attempt to increase the vitamin D activity of butter [Kon and Booth, 1934] would result only in an

increase in the orthodox vitamin D and not in the novel factor. This, in our experience [Kon and Booth, 1934] is actually the case.

Our finding that either butter or lard incorporated in the diet is less active than corresponding levels fed separately is not incompatible with the suggestion that a part of the antirachitic activity of these substances is due to their fatty acids. The antirachitic action of fat in the case of the rat must be due to the influence of the fat on the calcium and phosphorus metabolism, and it seems quite permissible to assume that the effect of one massive dose daily will be different from that of the same amount distributed over a period of 24 hours.

We have already mentioned that Zucker and Barnett [1922–23] suggest as an explanation of the mechanism of the action of fat the formation of insoluble calcium soaps. Recent work [Boyd *et al.*, 1932] would rather favour the view that the beneficial effect of fat is due to the creation in the gut of a p_H favourable to the uptake of minerals. We do not want to theorise on this point but wish to postpone judgment pending the collection by us of experimental data on the effect of various fatty acids on the calcium and phosphorus metabolism of the rachitic rat.

It would seem to us, that on the whole the arguments in favour of fat playing a rôle in the antirachitic activity of butter are quite serious enough to deserve careful consideration.

C. There is just a possibility that both fat and a novel factor are responsible for the antirachitic activity of the labile factor of butter and lard in the case of the rat. Insufficient experimental evidence prevents us at present from discussing this point any further.

We are led to believe that, unless the labile factor of butter is definitely proved to be antirachitic also for other species besides the rat, the only safe course for ascertaining the vitamin D activity of butter is to submit it to saponification and feed to rats the non-saponifiable residue only. Even then in order to decide *a priori* what might be the antirachitic value of such butter in human nutrition, it would be necessary to find out whether the orthodox antirachitic factor present therein is identical with irradiated ergosterol or with the vitamin D of cod-liver oil, or whether it is altogether different from these two.

We [Kon and Booth, 1933; 1934] have examined various butters by the widely used prophylactic method, making use of what is practically the official diet (the 2965 diet of Steenbock) and administering graded doses of butter separately from the diet, a method used to our knowledge by the majority of present day workers. We have very good reason to believe that, in the case of many butters, especially those collected in autumn and winter, values so found by this generally used technique for their alleged vitamin D content may be anything up to 4 or 5 times too high.

It may be also remarked that similar results were obtained by the line test technique as far as this is possible with butters of low potency [Kon and Booth, 1933; 1934].

We have purposely refrained from any discussion of the clinical antirachitic value of butter, but we think that our results may perhaps suggest a plausible explanation of the discrepancy of opinion existing between the clinician and the student of nutrition with regard to the antirachitic value of butter.

Summary.

1. When tested on rats, lard was found to possess an antirachitic activity of the same order as that of butter, a sample of home rendered lard being almost equal in that respect to winter and autumn butter, while a sample of American

lard was found to be fully as active as summer butter. As in the case of butter, the activity of lard is impaired by saponification, the degree of loss being greater in the case of the lard of lower activity.

2. Fatty acids prepared from various butters were found to exhibit a slight but definite antirachitic activity which could not be attributed to incomplete removal of the non-saponifiable residue. This activity was roughly proportional to the amount of fatty acid fed and was independent of the original activity of the butter.

3. When butter was incorporated in the diet, it was found to produce a lower antirachitic effect than that given by a similar amount of butter fed separately from the diet.

4. In the case of an autumn butter, which after saponification had lost a large part of its original antirachitic potency, this potency could be recovered practically completely when the administration of the non-saponifiable residue was accompanied by the feeding of the fatty acid incorporated in the diet.

5. Although the loss of potency observed by us in various samples of butter could be tentatively explained by assuming the existence of a new labile antirachitic factor, we are inclined to believe at present that this phenomenon could be at least as adequately, and certainly more simply, explained by the antirachitic effect on the rat of fat as such.

6. If this explanation is, as we believe, correct, then, unless the labile factor of butter is definitely proved to be antirachitic also for other species besides the rat, values obtained by accepted methods for the antirachitic activities of butters are about 4 to 5 times too high in winter and autumn and about twice too high in summer. On the other hand values obtained by direct feeding of highly potent butters giving complete protection at a daily level of 10–30 mg., such as irradiated butter or butter yielded by cows receiving large amounts of irradiated yeast, are correct.

7. On the same basis it is suggested that while the general antirachitic value of the labile factor is still in question the only safe course in ascertaining the vitamin D activity of butter is to submit it to saponification and feed to rats the non-saponifiable residue only.

We are indebted to Capt. J. Golding and Prof. H. D. Kay for helpful criticism; to the former also for the home-rendered lard; to Miss D. V. Dearden for the churning of the butters and to Mrs S. K. Kon for much help in the preparation of bones for analysis.

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XIX. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

IV. (*a*) THE OCCURRENCE OF CONJUGATED GLUCURONIC ACIDS IN THE ANIMAL BODY.

(*b*) OBSERVATIONS ON THE CONJUGATION OF *d*- AND *l*-BORNEOL.

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(*Received November 9th, 1933.*)

Glucuronic acid in normal animal urine.

Following the recognition of the part played by glucuronic acid in detoxication mechanisms, its possible occurrence in normal urine was suggested by Hoppe-Seyler [1882] and was further considered by Flückiger [1885] in the course of his investigations of the copper-reducing substances of human and animal urines. Several early observers [Haas, 1876 and others] had shown that normal human urine is slightly laevorotatory and it was probable that this laevorotation was due to the presence of conjugated glucuronic acids, which are all laevorotatory. Proof of the occurrence of glucuronic acid in normal urine was provided by Mayer and Neuberg [1900], who isolated it from human urine as the *p*-bromophenylhydrazine derivative. These workers concluded that glucuronic acid occurs in normal human urine conjugated mainly with phenol and cresol and to a lesser extent with indoxyl and skatoxyl. It had previously been suggested by Hoppe-Seyler [1882] that glucuronic acid might be excreted in normal urine as the conjugated indoxyl compound, and Mester [1888] found a laevorotatory and copper-reducing substance in the urine of dogs after administration of skatole, which he regarded as the hypothetical skatoxylglucuronic acid. Neuberg and Niemann [1905] also suggested that urea-glucuronic acid, which they had synthesised, might occur in normal urine, and Pollecoff [1924] stated that glucuronic acid is a probable constituent of urochrome, the yellow pigment of urine, isolated from human urine and carefully purified. Tollens [1910] has emphasised the fact that man excretes twice as much conjugated glucuronic acid daily as ethereal sulphate and even within the limits of normal behaviour much more glucuronic acid can be excreted daily than of ethereal sulphate. The quantity of glucuronic acid in normal urine has been determined by several workers and values from 4 to 25 mg. per 100 ml. have been quoted (see Table I).

With reference to the occurrence of glucuronic acid in the urine of other animals little can be found in the literature. Hagemann [1882] states that it is probably a part of the reducing substance he obtained from horse urine, while Kawanishi [1932] has estimated it in rabbit urine (see Table I). We have detected

Table I.

Observers	mg. glucuronic acid per 100 ml. urine: Normal human urine	Rabbit urine
Boku and Kin [1931]	13	—
Kawanishi [1932]	5 to 10	4 to 15
Mayer and Neuberg [1900]	4	—
Sauer [1930]	15 to 19	—
Tollens and Stern [1910]	25	—

glucuronic acid in the normal urine of all animals tested, using the Tollens naphthoresorcinol test, and we have confirmed its presence in human urine by the same test (see Table II).

Table II.

Animal	No. tested	Naphthoresorcinol test
Man	15	+
Dog	30	+
Cat	18	+ + +
Mouse	6	+ +
Guinea-pig	2	+

It is interesting to note that cat urines always give a much stronger positive test than the urine of other animals listed, and this strong test is associated with an intense Obermayer indoxyl reaction. It would seem probable that cat urine normally contains a relatively greater amount of the hitherto undescribed indoxylglucuronic acid than the urine of other animals tested, and it is therefore a likely source for isolating this compound. The conjugated glucuronic acids of normal urine (*i.e.* phenyl, cresyl, indoxyl and skatoxyl) have not been isolated from such urine, but phenylglucuronic acid has been isolated from the urine of sheep after administration of phenol [Salkowski and Neuberg, 1906]; cresylglucuronic acid has been isolated as a double barium salt with phenylsulphuric acid from the urine of dogs fed with *p*-cresol [Neuberg and Kretschmer, 1911]; and Neuberg and Schwenk [1917] have isolated a double barium salt of indoxylglucuronic and -sulphuric acids from urine after the feeding of indole. It is of interest to point out that the non-fermentable sugar of urine, which was shown by West *et al.* [1932] to increase during intestinal stasis in the dog, is probably in part glucuronic acid, which itself is not fermented by yeast.

Glucuronic acid in normal animal blood.

Scanty reference has been made in the literature to the occurrence of glucuronic acid derivatives in blood, but their presence there follows from their elimination by the kidney, since it is shown that the kidney does not synthesise conjugated glucuronic acids [Hemingway *et al.*, 1934]. Glucuronic acid, as the *p*-bromophenylhydrazine derivative, has been isolated from bovine blood by Mayer [1901], who states that it is probably constantly circulating in human blood. Letsche [1907] states that amongst the reducing substances in blood of carbohydrate nature are glucose and glucuronic acid; the presence of the latter was deduced from orcinol- and phloroglucinol-HCl tests and from the isolation of a hydrazone. Stepp [1919] showed it to be present in normal human blood by means of the naphthoresorcinol test, and Ørskov [1932], in a study of the ether-soluble acids of blood, claims that minute quantities of conjugated glucuronic acids are present. Lépine and Boulud [1905; 1906] have made estimations of the conjugated glucuronic acid in arterial and venous blood, in serum

and in the corpuscles, and find as much as 14 to 47 % of the blood-sugar to be conjugated glucuronic acids; they base their values on reducing powers and rotations, but, owing to interfering substances (now known to be present in blood) which affect these methods, these values cannot be accepted without further investigation.

We have confirmed the fact that human blood contains glucuronic acid, using the naphthoresorcinol reaction and find that dog and cat bloods also give a slight positive test. The test was carried out on blood after deproteinising with sodium tungstate and sulphuric acid. The test was then compared with a blank using the deproteinising agents and water and with a known positive test on blood containing bornylglucuronic acid. We have also found that the blood

Table III.

Animal	Method	Observer
Man	Naphthoresorcinol test	Stepp [1919] and this investigation
Dog	,,	This investigation
Cat	,,	,,
Ox	Isolation of *p*-bromophenylhydrazide	Mayer [1901]

and serum of man and the dog give very intense naphthoresorcinol tests for glucuronic acid, 10 minutes after oral administration of 2 g. of borneol, from which it seems that the detoxication of borneol begins to take place rapidly.

The stereochemistry of bornylglucuronide in relation to its formation in the body.

All natural glycosides possess the β-type of linkage, and this may now be regarded as true also for glucuronides synthesised *in vivo*. Bornylglucuronide, from the urine of dogs fed with borneol, has been shown by Hämäläienen [1911] to be a β-glucosidic compound, since it is hydrolysed by the β-glucosidase of emulsin. However, Miwa [1932] stated that not all β-glucuronides are hydrolysed by this β-glucosidase. Positive results have been obtained by Neuberg and Niemann [1905] with phenylglucuronide and euxanthic acid, by Hämäläienen [1911] with *l*-camphorylglucuronide, by Hildebrandt [1905] with syringa- and vanillin-glucuronide, by da Cruz [1930] with menthylglucuronide, and by Ishidate [1929] with *d*-camphorylglucuronide and menthylglucuronide. Negative results were obtained by Schmiedeberg and Meyer [1879] with *d*-camphorylglucuronide, by Sera [1914] with orcinol- and vanillin-glucuronides, and by Miwa [1932] with menthylglucuronide. The varying behaviour of emulsin towards conjugated glucuronic acids is explained by Miwa [1932] to be partly due to the activity of the enzyme which seems essentially dependent on method of preparation and degree of ageing. In nature all derivatives of glucose are without exception derivatives of *d*-glucose, and the naturally formed glucuronides also fall into this category. Bornylglucuronide on hydrolysis gives *d*-glucurone and *d*-glucuronic acid. These facts therefore show that the glucuronide present in urine after ingestion of borneol is β-bornyl-*d*-glucuronide. However, there still remains to be considered the optical configuration of the bornyl residue and its influence, if any, on conjugation.

It was found by Magnus-Levy [1907] that when pure *d*- or *l*-borneol was fed separately to dogs or rabbits, pure *d*- or *l*-bornylglucuronide was excreted in the urine and conjugation took place equally well with either isomeride. The conjugated glucuronic acids were isolated as the sodium salts (sodium β-*d*-bornyl-*d*-glucuronide, $[\alpha]_D - 36{\cdot}7^\circ$ in water, sodium β-*l*-bornyl-*d*-glucuronide, $[\alpha]_D - 66{\cdot}7^\circ$

in water). We have fed pure *l*-borneol[1] to dogs and have recovered from the urine pure *β*-*l*-bornyl-*d*-glucuronide, $[\alpha]_{5461} - 74{\cdot}6°$ in water. On the other hand, as is shown in Table IV, we find that when a mixture of *d*- and *l*-borneol is fed to dogs preferential conjugation of the *d*-isomeride occurs, and da Cruz [1930], on feeding inactive menthol to dogs found the menthol after liberation from the conjugated derivative to be optically active. In the course of many of our feeding experiments the rotations of the borneol fed, the glucuronide excreted and the borneol recovered from it after hydrolysis were noted (Table IV).

Table IV.

	$[\alpha]_{5461}$	% *d*-isomeride	
Borneol fed	+20° (EtOH)	73·7	
Bornylglucuronide excreted	−47·9 (water)	86·7	Mean increase
Borneol recovered after hydrolysis	+30 (EtOH)	85·5	12·4 %
Borneol fed	+26·5 (EtOH)	81·4	
Bornylglucuronide excreted	−45·6 (water)	93·0	Mean increase
Borneol recovered after hydrolysis	+33 (EtOH)	89·3	9·8 %

	Calculated from figures quoted by Quick [1927]	
	$[\alpha]_D$	% *d*-isomeride
Borneol fed	+5°	56·7
Bornylglucuronide excreted	−48	66; increase 9·3 %
Borneol recovered after hydrolysis	No observation	No observation

SUMMARY.

1. A brief summary including new observations is given of the normal occurrence of glucuronic acid in the urine and blood of various animals.

2. A stereochemical study of the conjugation of *d*- and *l*-borneol with glucuronic acid *in vivo* reveals a preferential conjugation of the *d*-isomeride.

The expenses of this work were in part defrayed by a grant from the Medical Research Council. One of us (R. T. W.) is indebted to the Council for a whole time assistance grant.

[1] *l*-Borneol fed had $[\alpha]_{5461} - 42{\cdot}7°$ in alcohol; Mitchell [1925] gives the rotations of *d*- and *l*-borneol as $[\alpha]_{5461} \pm 42{\cdot}2°$ in alcohol.

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XX. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

V. THE SITE AND MECHANISM OF THE FORMATION OF CONJUGATED GLUCURONIC ACID.

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(*Received November 9th, 1933.*)

When a substance which is metabolised with difficulty is introduced into the animal body several detoxication mechanisms can come into play, and it is well known that glucuronic acid, glycine, glutamine [Thierfelder and Sherwin, 1915] and sulphuric acid play various rôles in this connection. A scrutiny of the work for the last half century on the metabolism of certain compounds reveals that many of these (*e.g.* certain aliphatic alcohols, phenols, aldehydes, ketones, hydrocarbons, terpenes, aromatic acids, certain amines and heterocyclic nitrogen compounds) can be detoxicated by conjugation with glucuronic acid. This process of conjugation only occurs if the toxic compound carries a hydroxyl group, a $C_6H_5.COOH$, or a $C_6H_5.\overset{|}{C}.COOH$ group, or can form such a group by oxidation (or in some cases by reduction) in the organism. Thus, terpenes are oxidised to the corresponding alcohol and the latter conjugated with glucuronic acid for excretion [Fromm and Hildebrandt, 1901]:

$$C_{10}H_{16} \rightarrow C_{10}H_{15}OH \rightarrow C_{10}H_{15}O.C_6H_9O_6 \text{ (Terpinolglucuronide).}$$

Benzene is oxidised to phenol which is excreted conjugated with glucuronic and sulphuric acids [Braunstein *et al.*, 1931]. Toluene is oxidised to benzoic acid which is excreted conjugated with glycine and glucuronic acid [Epstein and Braunstein, 1931]. Chloral is reduced to trichloroethyl alcohol which is excreted as trichloroethylglucuronic acid [*cf.* Mering, 1882].

In the second stage of the detoxication the toxic body may combine with glucose to form an intermediate glucoside which is then oxidised to the glucuronic acid, or it may combine directly with preformed glucuronic acid. The former possibility was proposed by Fischer and Piloty [1891] as the mechanism by which conjugated glucuronic acids were formed in the animal, but it had then no experimental basis. None the less it has been widely accepted as an established fact [see Armstrong, 1919; Tollens, 1914]. Hildebrandt [1905; 1909] has claimed that on subcutaneous injection of coniferin (coniferylglucoside), syringin (methoxyconiferylglucoside) and bornylglucoside into rabbits the corresponding conjugated glucuronic acids were excreted in the urine. This observation has alternative explanations, either direct oxidation of the glucoside to the conjugated glucuronic acid, or previous hydrolysis of the glucoside in the body to give a free aglucone which combines with preformed glucuronic acid.

These considerations raise two questions: (1) What organ in the body

carries out the detoxication? (2) Is glucoside formation an intermediate stage in the detoxication process? With a view to providing an answer to these two questions it was decided to perfuse, by means of a pump-lung preparation, the isolated kidney alone and in conjunction with other isolated organs, and to investigate the reaction of such perfused organs to glucuronogenic and other substances added to the circulating blood.

Methods.

Four combinations of perfused tissues were used during these investigations and they are briefly described in the order in which they were employed.

(i) *The pump-lung-kidney preparation* (*Preparation A*).

The arrangement for these experiments was essentially the same as those previously described by Hemingway [1931; 1933]. The preparation was made from two dogs. The first, which weighed 16–18 kg., was anaesthetised, and about 500 ml. of blood were taken from the carotid artery. The blood was whipped, twice filtered through fine muslin and, after measurement of the volume, introduced into the apparatus.

A cannula was tied into the pulmonary artery after opening the chest wall and perfusion of the lungs commenced after removal from the thorax.

Meanwhile the second dog was anaesthetised and about 300 ml. of blood were removed. Saline was injected intravenously to replace this blood. The abdomen was opened in the middle line, the left kidney freed from its attachments and the vessels dissected. A cannula was tied in the ureter, the kidney removed and attached to the perfusion apparatus.

(ii) *The pump-lung-kidney-liver preparation* (*Preparation B*).

A liver, perfused by a method analogous to that employed by Bauer *et al.* [1932] was added to the perfused kidney preparation. The arrangement of the apparatus is shown in Fig. 1. From a large reservoir *R*, blood was taken by two pumps, P_1 and P_2, to supply respectively the renal and hepatic arteries (*R.A.* and *H.A.*) and a constant level reservoir, *P.R.*, which fed blood to the portal vein (*P.V.*). The kidney and the liver were supported on Büchner funnels the bottoms of which were covered with fine muslin filters. The blood which issued from the cut ends of the renal and hepatic veins was eventually gathered into a common funnel, *F*, again filtered and after perfusing the pulmonary system, collected in the main reservoir. The output of the pump P_1 was adjusted to maintain a pressure of 120–130 mm. Hg in the renal and hepatic arteries, and that of P_2 to keep the portal vein reservoir overflowing. The level of this reservoir was 10–15 cm. above the portal vein.

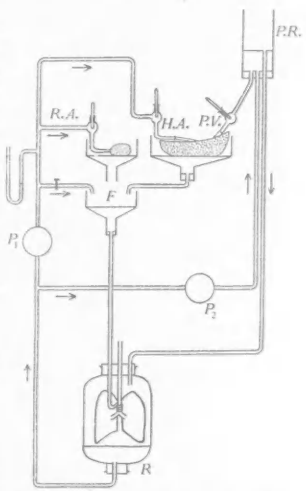

Fig. 1. Arrangement of apparatus in Preparation B.

The main reservoir and the Büchner funnels supporting the organs were placed in thermostats while the tubes conveying blood to and from the main reservoir and the lungs were water-jacketed with water circulated from one of the baths by an "autopulse" pump.

The preparation was made from two dogs and the technique of isolating the organs was identical with that described in the previous section up to the point of removing the kidney. Prior to this, the portal vein was dissected and two loose ligatures placed round it after cutting the splenic and gastric tributaries between ligatures. The hepatic artery was isolated. The aorta was clamped proximal to the renal arteries, the left kidney removed and perfusion commenced.

The portal vein was then cannulated and the liver washed with about 300 ml. of warm saline. The vein and the hepatic artery were clipped and the liver removed. A cannula was tied into the hepatic artery and after filling both cannulae with blood and attaching the appropriate tubes, perfusion was commenced. The first 200 ml. of blood coming from the liver was collected, whipped and filtered before being returned to the blood reservoir.

(iii) *The pump-lung-kidney-spleen preparation* (*Preparation C*).

The spleen vessels were dissected and loose ligatures placed round them. The kidney was removed and perfusion commenced, after which the spleen was removed and a cannula tied in the artery. The organ was then perfused.

(iv) *The pump-lung-kidney-limb preparation* (*Preparation D*).

The hind limb was selected for perfusion. The femoral vessels were dissected and loose ligatures placed round them and round the common iliac artery. The perfusion of the limb was commenced before removing the kidney, so minimising circulatory disturbances in the limb. A cannula was inserted into the femoral artery after tying the common iliac artery. The limb was washed through with defibrinated blood and, after removal by cutting through tissues and bone just below the head of the femur, perfusion was commenced. The blood issuing from the cut end of the femoral vein was collected in a funnel and led to *F*.

In all the experiments, with the exceptions mentioned below, the required amount of the substance whose conjugation was to be tested was dissolved in a small volume of saline and slowly added to the blood passing to the lungs, through funnel *F*.

Experimental.

(*a*) *The site of conjugation of glucuronogenic substances.*

The first organ to be considered was the kidney, since detoxication by conjugation with glycine to form hippuric acids is performed by this organ [Quick, 1932]. It seemed therefore possible that the kidney might perform a like function in regard to glucuronic acid.

As a preliminary it was necessary to demonstrate that the isolated perfused kidney was capable of eliminating preformed conjugated glucuronide. Preparation A was used in this investigation. It should first be stated that normal urine from the dog gives a slight positive test for glucuronides with naphthoresorcinol.

Our tests were carried out as follows: 2 ml. of urine, 2 ml. conc. HCl and 0·5 ml. of naphthoresorcinol (1 % in alcohol) were heated in a large test-tube for 2 mins. in boiling water. The tube was then removed from the water-bath and allowed to stand for 4 mins., and then cooled for 4 mins. in running water. The pigment was extracted with 10 ml. ether. Our experience is that this colour test for glucuronic acid, originally devised by Tollens [1908], although not a strictly quantitative test, is capable of detecting increments of glucuronic acid of the order of 0·25 mg. in 2 ml. urine, and that such increments can be compared colorimetrically.

Urine formed by an isolated kidney, when perfused with normal blood, also gives a positive test. It is, however, much fainter than that of normal urine, and

the intensity of the test falls to an almost zero constant value as the excretion proceeds. The urine from the isolated kidney was collected until it gave this almost zero constant value. At this point blood taken from a dog, which had been previously fed with borneol and which was known to be excreting glucuronide, was added to the circulating blood in the preparation. The result of the experiment is shown graphically in Fig. 2. It will be seen that the addition

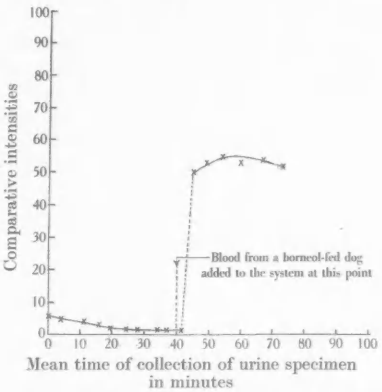

Fig. 2. Graph showing the effect of the addition of blood from a dog fed with borneol, on the intensity of the naphthoresorcinol test performed on urines excreted by an isolated perfused dog kidney. The intensity of the test performed on the bladder urine of the borneol-fed dog is taken as 100 arbitrary units of intensity.

of "borneol-blood" to the circulation of the pump-lung-kidney is followed by an immediate and marked increase of glucuronide in the urine formed by the kidney.

The next step in the investigation was to determine whether or not conjugation occurred in the isolated perfused kidney. The addition of phenol to the perfusing blood (50 mg. in 5 ml. saline to 570 ml. blood) was not followed by any increase in the glucuronide excretion, although the presence of free phenol was readily demonstrated in the urine formed within 5 mins. of the addition of the phenol to the blood. A similar experiment was performed with borneol, but in this case, owing to its low solubility, the crystalline solid was suspended in the circulating blood inside a fine muslin bag. Results as with phenol were negative.

The foregoing experiments showed that preformed conjugated glucuronic acid could readily be eliminated by the isolated perfused kidney, although the negative results in regard to conjugation left the site of formation an open question.

Embden and Glässner [1903] have shown that perfusion of the liver with blood containing phenol results in an increase of glucuronic acid in the perfused blood. A few workers have shown that there is a decreased glucuronic acid output when the liver is injured or non-functional owing to disease [Nazarjanz, 1931; Boku and Kin, 1931]. These experiments suggest that the liver is probably the site of synthesis. The results now to be described prove that this is so and also suggest that the liver alone performs this detoxication process. Preparations B, C and D were used in the series of experiments which lead to this

conclusion. The course of each experiment was similar to that already described in reference to the use of Preparation A. The following substances, all of which are known to form conjugated glucuronides in the intact animal, were added to the perfusion system: phenol, chloral hydrate, camphor and borneol.

(1) *Phenol.* This was added to the blood in two successive lots, (*a*) 30 mg. in 3 ml. normal saline, and (*b*) 70 mg. in 7 ml. normal saline. Within 4 mins. of the addition of each quantity of phenol, using the naphthoresorcinol reaction, marked increases in the glucuronide excretion were observed, (*b*) giving a considerably stronger result than (*a*). 14–15 mins. after the addition of (*b*), the glucuronide elimination by the kidney had sunk almost to zero, so that the process of conjugation and elimination was rapidly completed.

(2) *Chloral hydrate.* In the case of this substance the course of elimination of the conjugated glucuronide is given graphically in Fig. 3, which is constructed

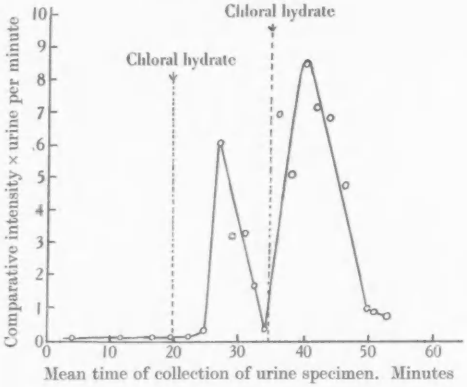

Fig. 3. Graph showing the elimination of glucuronic acid in the pump-lung-kidney-liver preparation in response to added chloral hydrate.

by multiplying the relative intensity (employing arbitrary units) of the naphthoresorcinol test by the rate of urine formation. As before the chloral hydrate was added in two lots, (*a*) 0·19 g. and (*b*) 3·03 g. each in 10 ml. normal saline (to a blood-volume of 1020 ml.) and the subsequent naphthoresorcinol reaction reached a maximum intensity in 5 to 8 mins. after each addition.

It will be observed that the second dose, some 15 times the amount of the first, did not produce a proportional increase in the glucuronide elimination, which no doubt implies some time limitation of the capacity of the liver to conjugate the chloral. It is also clear that, under the conditions of the experiment, only a part of the added chloral hydrate is eliminated *via* the kidney as conjugated glucuronide.

(3) *Camphor.* Camphor was added to the perfusing blood (1070 ml.), (*a*) in 20 ml. of saline which had previously stood in contact with solid camphor for 24 hours at 37°, and (*b*) by placing solid camphor in a fine muslin bag in the circulating system (at *F*, Fig. 1) so that the blood percolated the solid. In both cases marked increases in the glucuronide excreted were observed.

(4) *Borneol.* Solid borneol was percolated by the circulating blood (1015 ml.) as in (3) above. Positive results were again obtained.

In some early experiments the dogs used were initially under chloralose

anaesthesia. In all such cases the urine first formed in the liver-kidney perfusion system gave a marked indication of the presence of conjugated glucuronide. Chloralose, a condensation product of chloral and glucose, contains free chloral which (see (2) above) forms conjugated glucuronide in Preparation B. Chloralose anaesthesia was therefore abandoned after these early experiments.

Although the foregoing experiments proved that the liver is one of the sites of conjugation of glucuronic acid, the possibility remained that other organs might perform a like function. To test this possibility Preparations C and D were devised. The technique used was the same as that employed with Preparation B using phenol as the potential glucuronogenic substance. One experiment was performed with Preparation C, two with Preparation D, and in none of these was any conjugated glucuronide formed.

(*b*) *The mechanism of conjugation of glucuronogenic substances.*

(1) *Experiments with glycosides.* If the glycoside is an intermediate compound in the detoxication process then perfusion of the liver with blood containing glycosides should result in the formation of conjugated glycuronic acids. A second series of experiments was therefore made with Preparation B. In these (1) β-phenyl-*d*-glucoside and (2) β-bornyl-*d*-glucoside were added to the perfusing blood in separate duplicate experiments. The β-isomerides were used since all conjugated glucuronides formed *in vivo* are of the β-glycosidic type. The former glucoside was synthesised by the method of Fischer and Armstrong [1901], the latter by that of Fischer and Raske [1909]. β-Phenyl-*d*-glucoside (105 mg. in 10 ml. saline) was added to the blood (1035 ml.) in the perfusion system, whilst in the case of β-bornyl-*d*-glucoside the crystalline solid (65 mg.) was percolated as before by the blood (775 ml.). At no stage in either experiment after the addition of the glucoside was any increased elimination of glucuronide observed, although the conditions employed were identical with those giving positive results with known glucuronogenic substances. It should be added that in the case of β-phenyl-*d*-glucoside, the unchanged substance was found in the urine within 5 mins. of its addition to the perfusing blood. No free phenol was detected.

Another experiment was performed in which β-bornyl-*d*-galactoside (synthesised by the method of Robertson [1929]) was added to Preparation B. No evidence of the formation of conjugated galacturonic acid was obtained.

(2) *The influence of cyanide on the conjugation process.* The process of conjugation may require the cellular integrity of the liver or, on the other hand, it may depend on the existence of a preformed enzyme. In the former event one might expect the process of conjugation to be inhibited or abolished by cyanide. A suitable experiment, performed with Preparation B in which KCN (0·003 *M*) was added to the perfusing blood, confirmed this expectation, since added phenol was eliminated in the urine unchanged and no glucuronic acid was formed.

SUMMARY.

1. A description is given of a new technique for perfusing various organs (liver, spleen, tissues of the hind limb) in combination with the kidney.

2. It is demonstrated by using such preparations that the main, if not the only, site of formation and conjugation of glucuronic acid is the liver. The process of conjugation is abolished by cyanide.

3. No support is found for the hypothesis that the production of a glucoside is an intermediate stage in glucuronide formation.

The cost of this investigation was defrayed in part by grants from the Royal Society and from the Medical Research Council. One of us (R. T. W.) is indebted to the Medical Research Council for a whole time assistance grant.

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XXI. GLYCEROPHOSPHORIC ACID.

I. THE RECOGNITION AND SEPARATION OF α- AND β-GLYCEROPHOSPHATES.

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(*Received November 14th, 1933.*)

Synthetic glycerophosphates have been known for many years and have been prepared in several ways. The acid may exist in two isomeric forms, α and β. Carré [1903] showed that glycerol when esterified with phosphoric acid at 105° yielded almost entirely monoglycerylphosphoric acid. At higher temperatures further condensation took place. Later Power and Tutin [1905] prepared synthetic glycerylphosphates by a similar method and also obtained the mono-derivatives. Poulenc Frères [1907] showed that a well-crystallised sodium glycerylphosphate could be prepared by heating monosodium phosphate with two molecular proportions of glycerol and hydrolysing the resulting di-ester with sodium hydroxide. All these methods yielded mixtures of α- and β-glycero-phosphates.

There are three principal methods for preparing pure α- and β-glycerophos-phates. (1) King and Pyman [1914] mixed pure α-monochlorohydrin and tri-sodium phosphate, and on working up the product obtained pure α-glycero-phosphate. Pure β-glycerophosphate was obtained from α-dichlorohydrin and phosphoryl chloride. (2) Bailly [1915] prepared pure α-glycerophosphate from epichlorohydrin. (3) Fischer and Pfähler [1920] prepared pure α-glycero-phosphate from acetone-glycerol, phosphorus oxychloride and quinoline.

The following methods have been advanced to distinguish between the α- and β-isomerides.

1. Grimbert and Bailly [1915] found that the α-ester on careful oxidation (so as to avoid hydrolysis) yields a compound containing a ketone group, which can be identified by the Denigès reaction. The β-form on the other hand does not give this reaction.

2. Karrer and Salomon [1926] described a method which depends on the fact that barium β-glycerophosphate forms with barium nitrate a difficultly soluble double salt which precipitates on standing. The α-form does not give this double salt. Kay [1931] has criticised Karrer and Salomon's results on the ground that they used supersaturated solutions from which a salt would crystallise out on standing without the addition of barium nitrate. We have, however, put this method to an important use to be described later.

3. Since Levene and Yawagama [1920] had found that the rate of hydrolysis of the phosphoric esters of the sugars with sulphuric acid varied with the position of the phosphate group, it was thought advisable to see if the α- and β-forms of

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glycerophosphate would show this peculiarity. To test this point solutions of the α- and β-barium glycerophosphates were hydrolysed with *N* sulphuric acid at 100°. *k* was calculated for a unimolecular reaction. The average values for one experiment were for the α, $k=0{\cdot}00281$, and for the β, $k=0{\cdot}00266$. From these results it is evident that the rates of hydrolysis of the two isomerides with strong acids are not appreciably different.

4. *The enzymic hydrolysis method* for distinguishing between the two isomerides depends upon the fact that both animal and plant phosphatases hydrolyse the two forms at markedly different rates. Kay [1926] has shown that mammalian kidney phosphatase attacks the β- more rapidly than the α-isomeride. Earlier workers had stated that the α- and β-forms were attacked at the same rate. A plant phosphatase was investigated by a group of Japanese workers [Akamatsu, 1923; Inouye, 1928; Kobayashi, 1927; Asakawa, 1929], who showed its presence in takadiastase. It has been found by Kay and Lee [1931] that with most mammalian phosphatases and with takaphosphatase the β- is attacked more rapidly than the α-form. This fact has been made the basis for their method of distinguishing between the two isomerides. With phosphatase from *Propionibacterium jensenii* [Werkman and Kendall, 1931] and with blood-phosphatase, as shown by Roche [1931], the α- is attacked more rapidly than the β-form.

In the present investigation a study has been made of the rates of enzymic hydrolysis of pure β- and of a specially purified α-glycerophosphate. Purification of the α- from small amounts of the β-isomeride has been accomplished both by the use of Karrer's double salt method and by partial enzymic hydrolysis of the impure material. Since the β-isomeride is hydrolysed more rapidly than the α-form, the former is removed and although there is some loss of the α-form, it is obtained pure towards the end of the reaction.

In the expectation that the α-glycerophosphate might consist of two optical enantiomorphs with different rates of enzymic hydrolysis, an attempt was made to effect a separation of the one enantiomorph from the other by means of the partial enzymic hydrolysis of purified α-glycerophosphate. An attempt was also made to resolve the α-form by means of fractional crystallisation of several of its alkaloidal salts. None of the fractions obtained by the method of partial hydrolysis or by fractional crystallisation appeared to have any significant degree of optical rotation. In a second communication [Rae, 1934], the results are presented of a study of the relative amounts of the α- and β-isomerides in the glycerophosphates derived from the naturally occurring phosphatides.

Experimental.

Materials. The crystalline salt of sodium β-glycerophosphate, obtained from Boot's Pure Drug Company, appeared to consist almost entirely of the β-isomeride. It gave only a very slight Denigès reaction, and no sample of β-glycerophosphate has been obtained or prepared in this laboratory which has a higher rate of enzymic hydrolysis. On the other hand, several samples of the α-isomeride, which have been obtained from time to time, have appeared to contain different and widely variable amounts of the β-isomeride. Because of the discrepancies in the various experiments in which the reputedly pure α-compound was used, it was thought advisable to prepare some of the material from authentic α-monochlorohydrin by King and Pyman's [1914] method. For this purpose Eastman Kodak monochlorohydrin was purified by fractional distillation under reduced pressure until a fraction of constant boiling-point was obtained. 50 g. of this material were condensed with phosphoric acid, and 49 g. of calcium α-glycerophosphate were obtained, 52 % of the theoretical yield.

The results of several experiments with reputed α-glycerophosphate were communicated to Dr F. L. Pyman, of Boot's Pure Drug Company, who very kindly prepared a special batch of the α-salt, which appeared practically identical with that prepared in Toronto.

Methods of estimating rates of enzymic hydrolysis.

(1) *By animal phosphatase.*

Enzyme. Phosphatase occurs abundantly in liver, kidney, intestine and bone. For extraction the fresh tissue is ground up with 10–20 times its weight of chloroform water and allowed to stand for 48 hours at room temperature. It is then filtered through cotton-wool or coarse filter-paper and is ready for use.

Substrates. Approximately $M/100$ aqueous solutions of sodium glycerophosphate were used. These were prepared from the barium salts by adding an equivalent amount of sodium sulphate. The various substrates to be compared were analysed for phosphorus colorimetrically [King, 1932] and diluted to the same concentration.

Buffers. The optimum p_H for mammalian phosphatase acting on glycerophosphates is between 8·9 and 9·2. The following buffers were used in different experiments.

(i) Sørensen's glycine buffer, p_H range 8·53–12·90 [Clark, 1928].
(ii) Palitzsch's borate buffer, p_H range 6·77–9·24 [Clark, 1928].
(iii) Michaelis's veronal buffer, p_H range 6·8–9·6 [Michaelis, 1930].

Method. The hydrolyses are carried out as follows. A series of tubes at a range of p_H covering the optimum is prepared (4 for each p_H), using for each 5 ml. substrate (adjusted to the desired p_H), 5 ml. buffer and 1 ml. of the enzyme solution. To two of each set of 4 tubes (the controls) are immediately added 2 ml. of 25 % trichloroacetic acid; after 10 minutes the contents are filtered. The other 2 tubes are placed in a water-bath at 37·5° for exactly 2 hours. These are then removed, 2 ml. of 25 % trichloroacetic acid added and the contents filtered after

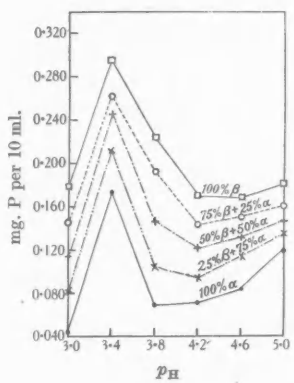

Fig. 1.

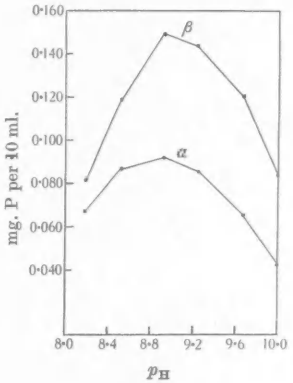

Fig. 2.

Fig. 1. Rates of hydrolysis by takaphosphatase of pure α-; 75 % α-, 25 % β-; 50 % α-, 50 % β; 25 % α-, 75 % β-; and pure sodium β-glycerophosphates. Phthalate buffers used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates. Glycine buffers used.

10 minutes. 10 ml. of filtrate from each tube are then analysed for inorganic phosphorus by a colorimetric method. The increase in inorganic phosphorus, expressed in mg. P per 10 ml. of reaction mixture, represents the amount of hydrolysis. In no case was the amount of hydrolysis a large fraction of the total substrate present.

(2) *By plant phosphatase.*

Enzyme. 0·5 ml. of 2 % takadiastase solution freshly prepared.

Buffers. The optimum p_H for plant phosphatase is 3·0–5·0.

(i) Clark and Lub's phthalate buffer, p_H range 2·2–6·2 [Clark, 1928].

(ii) Michaelis's [1931] acetate-veronal buffer, p_H range 2·62–9·64.

Substrates. These were the same as with mammalian phosphatase except that the p_H was adjusted to 4·2 before adding the buffer or the enzyme. This was the mean p_H of the several buffer mixtures used, and the final p_H of the mixtures did not differ appreciably from that of the buffer added in any case. The other details were the same as for the mammalian phosphatase.

Figs. 1 and 2 show the type of results obtained with pure α- and β-glycerophosphates using mammalian phosphatase and takaphosphatase.

Separation of the α-isomeride from the β-isomeride by enzymic means.

If pure α- or β-glycerophosphate is hydrolysed enzymically and fractions are removed from the reaction mixture as the hydrolysis proceeds, these fractions should show no difference in their rates of enzymic hydrolysis; but if a mixture of α- and β-glycerophosphates be used there should be a progressive change in the rates of hydrolysis of the fractions removed, since the β-form is hydrolysed faster than the α-isomeride. A large amount of sodium glycerophosphate was hydrolysed by the enzyme and portions of the reaction mixture were removed at the start and at approximately 25, 50 and 75 % hydrolysis. These fractions were freed from inorganic phosphorus by adding the calculated amount of magnesia

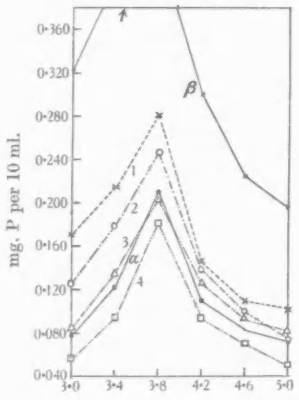

Fig. 3.

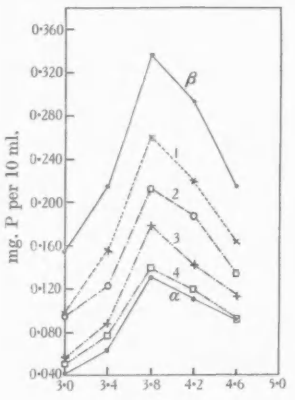

Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α-glycerophosphate (source 1) with takaphosphatase.

Fig. 4. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α-glycerophosphate (source 2) with takaphosphatase.

mixture without the ammonia. The solutions were then made pink to phenolphthalein by adding ammonia and the inorganic phosphate precipitated was centrifuged off. To the centrifugate was added an excess of normal lead acetate which precipitated the lead glycerophosphate. This was decomposed with hydrogen sulphide, and the solution, freed from this reagent, was made alkaline to phenolphthalein with baryta.

The barium glycerophosphate was precipitated by adding the filtrate to four volumes of alcohol. When two samples of reputed α-glycerophosphate, obtained from two different sources, were treated in this way and the several fractions compared for rates of enzymic hydrolysis, the results shown in Figs. 3 and 4 were obtained. These findings indicate that the α-salt as supplied to us contained some of the β-isomeride as an impurity.

Separation by double salt precipitation with barium nitrate.

An attempt was therefore made to purify commercial calcium α-glycerophosphate, after conversion into the barium salt, by a modification of Karrer and Salomon's [1926] method for differentiating the α- from the β-form. 30 g. of the barium salt dissolved in 300 ml. of water and 24 g. of barium nitrate dissolved in 300 ml.of water were mixed and allowed to stand in the refrigerator. The solution was reduced in volume until a precipitate had formed. After filtering, the filtrate was added to alcohol and the precipitated barium glycerophosphate was dissolved

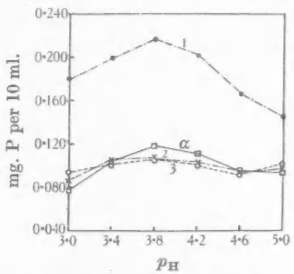

Fig. 5. Rates of hydrolysis by takaphosphatase of reputed sodium α-glycerophosphate before and after treatment with barium nitrate; (1) before treatment, (2) one treatment, (3) two treatments. α- refers to authentic α-acid.

in water and reprecipitated. This process was repeated to remove all the barium nitrate. The salt was again treated with the required amount of barium nitrate, and after standing, the barium glycerophosphate was again separated. The original salt and the salt after one, and after two, treatments were then compared as to their rates of enzymic hydrolysis. The results are given in Fig. 5. Judging by the rates of enzymic hydrolysis, one treatment with barium nitrate is sufficient to free the α-salt from the contaminating β-salt.

Attempts to separate α-glycerophosphate into optical enantiomorphs.

The method used above for estimating the relative amounts of α- and β-isomerides in a mixture of the two is open to a serious objection. Since the α-form has an asymmetric carbon atom in its formula, it might be expected that *d*-, *l*-, racemic and *dl*-, forms might all exist, and that these various forms might be hydrolysed at different rates. If this were so, what is considered from

the graph to be a mixture of the α- and β-isomerides might in fact contain no β-form but might be only the *d*- or *l*- form of the α-salt being hydrolysed at a faster rate than the authentic racemic α-salt. To settle this problem an attempt was made to resolve the synthetic α-form into its optical antipodes. Some earlier workers have suggested that the naturally occurring α-form was optically active. Levene and Rolf [1919] found slight activity in glycerophosphates from natural sources. The amount of rotation depended to a large extent on the method and the amount of purification used. Karrer and Benz [1926, 1, 2; 1927], after carefully separating the α- and β-forms from natural sources, obtained no rotation for either salt. They consider that earlier workers who found activity were probably observing the rotation of some impurity. Karrer and Benz, although they failed to obtain a separation with either strychnine or quinine, still believe that the α-form is optically active, because they prepared a dimethyl ether of the dimethyl ester of the α-salt which showed some slight activity. Abderhalden and Eichwald [1918] endeavoured to prepare the α-salt in its active form by acting on *d*-monobromohydrin dissolved in pyridine with phosphorus oxychloride in the cold. After a lengthy procedure, they separated the glycerophosphoric acid as the lithium salt. The analysis gave values too low in phosphorus and too high in carbon. The observed rotation was +0·48°, giving a specific rotation of +6·26°. In none of these attempts has conclusive evidence been given for the resolution of the α-salt.

The following attempts have been made to resolve the α-salt into its optical enantiomorphs.

(*a*) *By enzymic means.* Takaphosphatase was the enzyme used. The method was similar to that described above when the purification of the commercial α-salt was discussed. The three or four fractions obtained were analysed for phosphorus and barium, and their optical activity (Hg green) and their rates of enzymic hydrolysis were compared, using both mammalian and takaphosphatase. The results obtained when purified α-glycerophosphate or the synthetic product

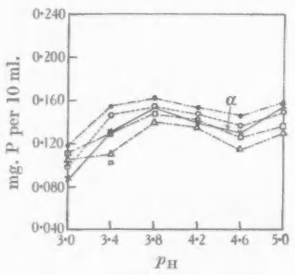

Fig. 6.

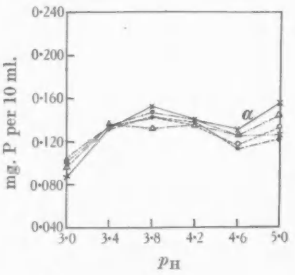

Fig. 7.

Fig. 6. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α-glycerophosphate purified by means of barium nitrate. The solid line is given by an authentic α-salt.

Fig. 7. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α-glycerophosphate prepared synthetically by the Pyman method. The solid line is given by an authentic α-salt.

was used are shown in Figs. 6 and 7. These graphs indicate that if any resolution has occurred, it is without effect on the rates of enzymic hydrolysis. The optical rotations were all less than the experimental error.

Propionibacterium jensenii contains a phosphatase which hydrolyses glycerophosphates at an optimum p_H of 7·0. To test the effect of this enzyme a large amount of these bacteria was obtained. By following a similar treatment to that described above for takaphosphatase, fractions were obtained at various percentages of hydrolysis, and these were compared as to their optical activity and enzymic activity. There was no significant difference between the fractions.

(*b*) *By the use of active alkaloids.* Brucine glycerophosphate was prepared by treating 10 g. of Ca α-glycerophosphate with sufficient *N* oxalic acid to form the free acid. The calcium oxalate was centrifuged off, and to the filtrate was added a slight excess of brucine dissolved in 50 % alcohol. The fractions which crystallised out were dissolved in 20 % alcohol, and a slight excess of barium acetate was added. The mixture was poured slowly into four volumes of alcohol and the barium glycerophosphate formed was filtered off. This salt was reprecipitated for purification. The fractions were analysed for phosphorus and compared as to rates of enzymic hydrolysis and optical activity. The results showed that, although there was a slight optical rotation in all fractions, there was no definite shift in values from one member of the series to the next. The results were with one or two exceptions within the experimental error. The resolution, if any, was without effect on the rates of enzymic hydrolysis.

A second experiment was done without converting the fractions into the barium salts. In this there was a slight shift in rotation, but since brucine itself has such a high negative rotation this might be due to varying traces of brucine.

Quinine glycerophosphate was made by mixing quinine sulphate with barium glycerophosphate. After filtering off the barium sulphate formed the mixture was allowed to crystallise spontaneously. The results from these fractions indicated that the optical rotations were not of sufficient magnitude to be considered significant. Quinine glycerophosphate was then prepared using the free alkaloid instead of the sulphate. Table I gives the results obtained.

Table I.

Fraction	Quinine glycero-phosphate g.	P %	$[\alpha]_D$	Enzyme experiments mg. P hydrolysed	
				Mammalian phosphatase	Taka-phosphatase
1	10·00	4·40	−0·4°	0·238	0·129
2	14·00	3·36	−0·1	0·236	0·142
3	16·00	3·42	−0·1	0·245	0·148
4	9·00	3·45	+0·1	0·269	0·145
5	11·00	3·30	0·0	0·261	0·142
6	7·05	3·44	0·0	0·241	—
7	7·96	3·35	−0·4	0·252	—
8	7·00	3·30	−0·2	0·229	0·132

These results do not show any decided drift of optical activity in either direction. Another experiment was done without conversion into the barium salt. The fractions obtained were analysed for nitrogen and phosphorus and their solubilities determined. There was no significant difference in solubility.

From these four attempts it was concluded that there had been insufficient evidence to indicate any resolution of the α-glycerophosphate into its optical antipodes by means of the active alkaloids quinine and brucine.

There remained the possibility that a resolution had occurred but that the optical rotations of the fractions obtained were too small to measure. An attempt was therefore made to obtain an increase in rotation by the addition of tungstates

and molybdates as Gernez [1887; 1888; 1889, 1, 2] had shown to be the case with tartaric acid. When a molecular proportion of sodium molybdate was added to dilute solutions of tartaric acid and α-glycerophosphoric acid the following results were obtained.

Table II.

Salt used	g. dissolved in 15 ml.	Observed rotation before	Observed rotation after	Increase in rotation
d-Tartaric acid	0·150	+0·4°	+13·33°	33·3 times
α-Glycerophosphoric acid	0·172	0	0	0

These results were obtained with Dr Pyman's authentic synthetic α-salt and two fractions from enzymic hydrolysis of the synthetic α-salt. A similar result was obtained using sodium tungstate in place of sodium molybdate. When two molecular proportions of either sodium molybdate or tungstate were used the result was the same. Apparently the addition of these salts to the glycerophosphates does not enhance the rotation, if there is such, to a point where it is detectable.

SUMMARY.

Various methods for determining the relative amounts of α- and β-glycerophosphates are described, and the application of one of these to unknown glycerophosphates is given in detail.

An enzymic method for detecting small amounts of the β-isomeride in reputed α-salts is described and a method outlined for the purification of the α-salt.

An attempt has been made to resolve the α-salt into its optical isomerides using an enzymic method and the alkaloids brucine and quinine. No positive evidence of separation has been obtained.

The addition of molybdates and tungstates does not appear to increase the rotation of glycerophosphoric acid to a point where it can be read.

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XXII. GLYCEROPHOSPHORIC ACID.

II. THE GLYCEROPHOSPHORIC ACID OF THE NATURALLY OCCURRING PHOSPHATIDES.

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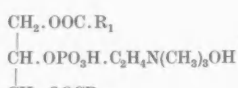
(*Received November 14th, 1933.*)

The naturally occurring phosphatides are usually represented as compounds of fatty acids and a base with the α- (unsymmetrical) variety of glycerophosphate (*e.g.* lecithin I), but the possibility has long been recognised that the β-variety (lecithin II) may also occur.

I



II

Glycerophosphoric acid can be obtained by hydrolysing the phosphatides with baryta. From this acid, as it occurs in egg-lecithin, liver-lecithin, brain-lecithin, kephalin and calcium phosphatidate, barium salts were obtained and these were investigated as to the relative amounts of the α- and β-isomerides present, using an enzymic method. Some objections as to the applicability of this method were removed.

Willstätter and Lüdecke [1904] found that the glycerophosphoric acid prepared from egg-lecithin was optically active, and they therefore concluded that it was the α-form. This observation may prove the presence of the α-form but does not exclude the possibility of the β-isomeride being present. Tutin and Hahn [1906] maintained that both the α- and β-forms were present. Grimbert and Bailly [1915] introduced a simple chemical test for differentiating the two isomerides which was based on the Denigès reaction, and using this test they showed that lecithin was a mixture of both varieties. Levene and Rolf [1919], using a highly purified egg-lecithin, obtained a glycerophosphate which had an optical rotation of $-0{\cdot}74°$. Willstätter and Ludecke's value was $-1{\cdot}71°$. Both groups of workers assert that the magnitude of the rotation depends very much on the mode of preparation.

Karrer and Salomon [1926] have shown, using a double salt precipitation method, that both varieties occur in lecithin. Owing to the confusion in this field and to the fact that Kay and Lee [1931] had recently brought forward a quantitative method for estimating the relative amounts of the α- and β-isomerides present in a mixture, it was thought advisable to prepare several phosphatides and estimate the relative amounts of the two isomerides in the glycerophosphates prepared from them.

[1] An abstract from a thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy in the University of Toronto.

Experimental.

Preparation of the phosphatides.

Lecithin was prepared from liver by a modification of Levene's [1921] method. 43 pounds of fresh ox-liver were minced and dried *in vacuo* at 37°. The material was finely ground and extracted with large volumes of alcohol. The extract was reduced in volume and allowed to stand overnight at 0°. This precipitated the cerebrosides. To the filtrate was added a saturated solution of cadmium chloride in hot ethyl alcohol until precipitation was complete. The crude lecithin cadmium chloride was repeatedly washed with ether to remove the kephalin. The purified salt was suspended in chloroform and decomposed with ammonia gas. After distilling off the chloroform the residue was dissolved in a minimum volume of ether and precipitated by adding it to a large volume of acetone. The yield was 69·8 g. Commercial egg-lecithin was purified by treating the material with cadmium chloride and recovering the lecithin in the same manner as that described above.

Brain-lecithin and brain-kephalin were prepared in the following way. 60 pounds of fresh ox-brain were freed from adhering tissue, minced and extracted with large volumes of acetone to dry the material and to free it from neutral fat. The brain was extracted with ether and the extract reduced to a small volume and allowed to stand at 0° for 15 hours. The supernatant liquid, now free from cerebrosides, was poured into alcohol. The alcohol-soluble part contained the lecithin. This was separated from the solution in a manner analogous to that described in the preparation of liver-lecithin. The kephalin was dissolved in ether and again allowed to stand at 0°, during which time more cerebrosides separated out. This process was repeated until no further precipitation occurred on standing. The liquid was added to alcohol and the kephalin precipitated. The yield was 455 g.

Chibnall and Channon [1927, 1, 2, 3] showed that the ether-soluble substances of cabbage-leaf cytoplasm contain little, if any, phospholipin in the commonly accepted use of the term, the major part of the lipin being present as the calcium salt of a diglyceridephosphoric acid. The calcium salt of phosphatidic acid was prepared in the following way. Four pounds of dried cabbage were exhaustively extracted with ether in a modified Soxhlet apparatus. The extract was evaporated to dryness, taken up in a small volume of ether and precipitated by adding to four volumes of acetone. The part insoluble in hot acetone gave the following results on analysis: P 4·00, 3·92 %; Ca 5·50, 5·45 %; Ca/P = 1·07. Calculated for Ca distearylglycerophosphoric acid: P 4·18 %; Ca 5·39 %; Ca/P = 1.

Preparation of barium glycerophosphate from the phosphatides.

The phosphatide was dissolved in a suitable solvent and emulsified by adding it slowly to an equal volume of hot water. This emulsion was then added to a hot baryta solution and boiled for one hour on the water-bath. The fatty acid formed barium soaps, and these were filtered off and washed with hot water. The filtrate and washings were treated with carbon dioxide to remove excess barium. The filtrate was reduced in volume and a slight excess of normal lead acetate was added. The lead glycerophosphate was washed, suspended in water and decomposed with hydrogen sulphide. The solution was freed from hydrogen sulphide and made neutral to phenolphthalein by the addition of baryta. It was then added to four volumes of alcohol, and the precipitated barium glycerophosphate was washed with alcohol and ether and dried *in vacuo* over sulphuric acid. In this

manner barium glycerophosphates were prepared from egg-, liver-, and brain-lecithin, brain-kephalin and calcium phosphatidate.

These salts were analysed for barium, using a micro-gravimetric method. This method makes use of the filter stick (Stäbchen) and the residue is weighed as barium sulphate. Another method used was the titrimetric benzidine method described by King [1932, 1]. The phosphorus was estimated colorimetrically, using King's [1932, 2] method.

Estimation of relative amounts of α- and β-isomerides in glycerophosphates from natural sources.

Both animal and plant phosphatases hydrolyse α- and β-glycerophosphates at different rates. It was decided, therefore, using Kay and Lee's [1931] enzymic method, to determine the relative amounts of the α- and β-isomerides of glycerophosphoric acid occurring in the natural phosphatides. Solutions of authentic

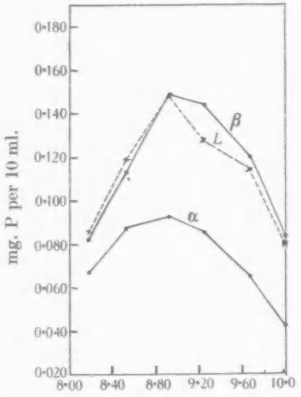


Fig. 1.

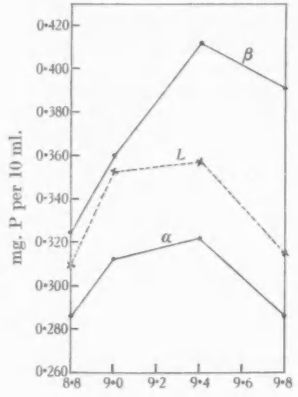


Fig. 2.

Fig. 1. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from egg-lecithin. Glycine buffer used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from liver-lecithin. Veronal buffer used.

α- and β-salts, and of the unknown glycerophosphate were made up approximately $M/100$, analysed for phosphorus and diluted to exactly the same strength. Rates of hydrolysis were measured by the method described in the previous paper [Rae *et al.*, 1934]. From these curves it may be concluded that the glycerophosphate from egg-lecithin (Fig. 1) is a mixture of α- and β-forms in which the β-form predominates. Liver-lecithin (Figs. 2 and 6) is a mixture of about equal proportions of α- and β-forms. Brain-lecithin (Fig. 3), brain-kephalin (Fig. 4), and calcium phosphatidate (Fig. 5) contain predominantly the α-form. The study of rates of enzymic hydrolysis thus furnishes a simple method for determining the relative amounts of the α- and β-isomerides occurring in the natural phosphatides.

The objection that since the α-form contains an asymmetric carbon, what is considered a mixture of α- and β-forms may be only one of the optical isomerides

of the α-form, has been dealt with in a previous paper [Rae *et al.*, 1934], where it has been shown that in several attempts to resolve the α-form, in no case were fractions obtained that exhibited any difference in their rates of enzymic hydrolysis.

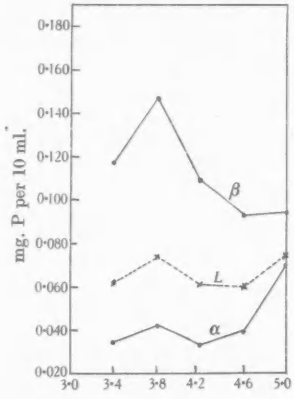


Fig. 3.

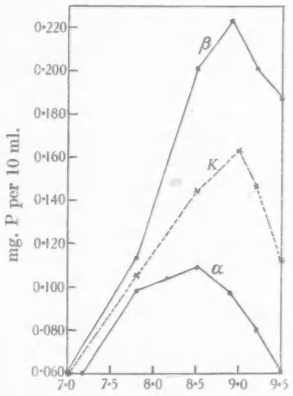


Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from brain-lecithin. Phthalate buffer used.

Fig. 4. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from brain-kephalin. Glycine buffer used.

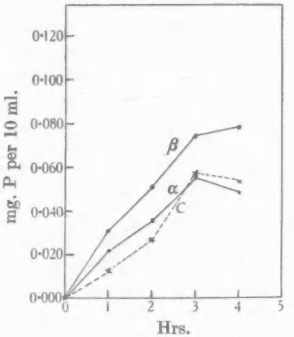


Fig. 5.

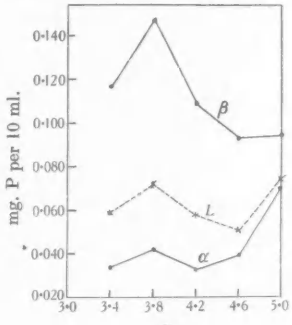


Fig. 6.

Fig. 5. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from calcium diglyceridephosphoric acid from cabbage. All at p_H 8·9.

Fig. 6. Rates of hydrolysis by takaphosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from liver-lecithin. Phthalate buffer used.

Another objection is that a wandering of the phosphate group from the α- to the β-position or *vice versa* may occur during the hydrolysis of the phosphatide with baryta. Thus a true picture of the relative amounts of the two isomerides present would not be obtained. To test this, samples of both α- and β-glycero-

phosphates were boiled for 2 hours with the customary amount of baryta. These salts were then compared enzymically and no change in the characteristic rate of hydrolysis was observed in either of them.

These results clearly indicate that the glycerophosphoric acid of the natural phosphatides consists of both the α- and β-isomerides. The formulae of these phosphatides should be written to represent the probability of the occurrence of the symmetrical β-variety, and not always as the asymmetrical α-type.

Summary.

The relative amounts of α- and β-glycerophosphates occurring in several natural phosphatides have been determined by an enzymic method. Egg-lecithin contains a glycerophosphoric acid which is predominantly the β-form; liver-lecithin contains about equal amounts of the α- and β-forms; whereas brain-lecithin, brain-kephalin and calcium phosphatidate contain predominantly the α-form.

My thanks are due to Dr E. J. King, who directed this research, and to Dr H. D. Kay, under whose direction some of the enzyme work was carried out.

This research was carried out under the tenure of a fellowship in the Department of Medical Research in the University of Toronto.

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XXIII. ON METHODS OF DETERMINING THE DEGREE OF CALCIUM DEFICIENCY OF SHEEP.

By ALLAN HENRY HECTOR FRASER, WILLIAM GODDEN and DOUGLAS WILLIAM AUCHINACHIE.

From the Rowett Research Institute, Bucksburn, Aberdeen.

(*Received November 11th, 1933.*)

The effect of lime and cod-liver oil supplements on the growth and serum-Ca of sheep fed on a Ca-deficient diet has been previously discussed by two of us [Auchinachie and Fraser, 1932].

The present communication records further experiments on the same subject in which Ca-balance determinations over a period were combined with blood analyses and clinical observations on the sheep. It emphasises the possible fallacies which may arise from any single method of investigation.

Four groups of five young sheep of similar age and size were fed the following basal ration:

Cooked maize	1 part	2 lbs. per diem.
Crushed oats	1 ,,	
Bran	1 ,,	
Dried distiller's grains	1 ,,	
Turnips	to 10 lbs. per head daily	
Oat straw	to ½ lb. ,,	
Water and salt (NaCl) *ad lib.*		

This was supplemented in the various groups as under:

Group I. Nothing.
II. 2·5 ml. cod-liver oil per head daily.
III. 2·2 lbs. of chalk per 100 lbs. concentrates.
IV. 2·2 lbs. of chalk per 100 lbs. concentrates + 2·5 ml. cod-liver oil per head daily.

The relative intakes of CaO on these diets are shown in Table I. Experimental feeding began on 27th October and continued until 24th May, a period of 209 days. The feeding pens were inside, covered, concrete-floored and bedded with moss litter.

Results.

Clinical.

The growth curves of the four groups are shown in Fig. 1. For a period of 84 days there was no differentiation in growth-rate. Subsequently growth-rate diminished in the basal group. In confirmation of previous work, a combined supplement of chalk and cod-liver oil had a greater effect on growth than either chalk or cod-liver oil alone; but in contrast to earlier findings the beneficial effect of chalk alone was greater than that of cod-liver oil alone [Auchinachie and Fraser, 1932].

The group food consumption was unequal. The question therefore arises as to how much of the basal group's growth retardation was due to decreased appetite, and how much to inefficient utilisation of the food consumed. The question can be answered by consideration of Fig. 2, where the weight of dry matter consumed per month in each group is plotted against time. The food consumed by group IV

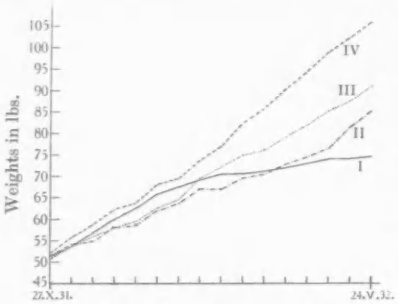

Fig. 1.

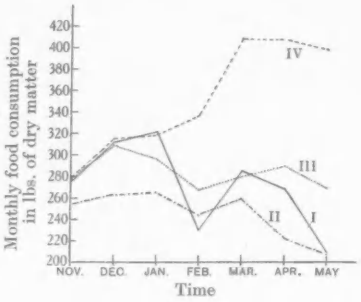

Fig. 2.

Fig. 1. Live weight increase (1931–32).

Group I. Basal. ————.
Group II. Basal + C.L.O. —·—·—.
Group III. Basal + $CaCO_3$ ·····.
Group IV. Basal + $CaCO_3$ + C.L.O. — — —.

Fig. 2. Food consumption.

Group I. ————. Group II. —·—·—. Group III. ·····. Group IV. — — —.

is seen to be almost double that of any other group. It may therefore be assumed that the omission of either chalk or cod-liver oil markedly decreased appetite. The relative amounts of food consumed during the balance experiments were slightly different owing to idiosyncracies of the individual animals and somewhat differing responses to cage environment.

One effect of the deficiencies of the basal ration was therefore to decrease appetite and food consumption. The utilisation of the food consumed was also inefficient, since while group I consumed more food than group II, its final average weight was nevertheless substantially less.

Balance determinations.

Balance determinations were made on one sheep from each group towards the close of the experiment when group differentiation was marked. The sheep were confined in metabolism cages over a period of 13–16 days. N, CaO and P_2O_5 balances were determined, and the results are shown in Table I. On the basal ration the daily intake of N was 12·18 g., of CaO, 2·10 g. and of P_2O_5, 7·20 g. Of this intake the sheep retained some 4 g. of N, some 0·08 g. CaO and some 1·4 g. P_2O_5. The sheep was therefore practically in equilibrium as far as calcium was concerned.

The chalk supplement increased the CaO intake to 7·20 g. It raised the CaO balance to 1·0 g., at the same time halving P_2O_5 retention and leaving the N balance unaffected.

Cod-liver oil raised the positive CaO balance to 1 g., but at the same time raised the P_2O_5, this latter effect being precisely opposite to that of chalk.

Table I. *Results of balance experiments with individual sheep.*

Group	Ration and average daily intakes (g.)	Average daily excretions: Nitrogen Urine g.	Nitrogen Faeces g.	CaO Urine g.	CaO Faeces g.	P_2O_5 Urine g.	P_2O_5 Faeces g.	Average daily balances: N g.	CaO g.	P_2O_5 g.
I	Basal N, 12·18; CaO, 2·10; P_2O_5, 7·20	4·32	3·79	0·0045	2·02	0·60	5·22	4·07	0·08	1·38
II	Basal+C.L.O. N, 14·57; CaO, 2·48; P_2O_5, 8·43	4·97	4·62	0·0044	1·46	3·38	2·84	4·98	1·02	2·21
III	Basal+Chalk N, 12·18; CaO, 7·20; P_2O_5, 7·20	4·46	3·58	0·0049	6·18	0·04	6·54	4·14	1·02	0·62
IV	Basal+Chalk+C.L.O. N, 15·56; CaO, 8·16; P_2O_5, 7·89	6·91	4·12	0·0211	6·82	1·04	6·07	4·53	1·32	0·78

Chalk and cod-liver oil together raised the CaO balance to the highest level, namely 1·3 g., and decreased P_2O_5 retention.

The relative excess of P_2O_5 retained in groups I and II raises an interesting problem. In group I with calcium in equilibrium there was a daily retention of 1·38 g. of P_2O_5. Obviously this could not have been deposited in the bones in the normal manner as calcium phosphate, and yet osseous tissue is the only large storage depot for phosphorus in the body. There is no evidence in the experimental data to elucidate this point, but further investigations are in progress along these lines.

Blood analysis. The results of periodical serum-Ca and blood-inorganic P determinations are shown in Table II. The changes in plasma-phosphatase have been previously recorded [Auchinachie and Emslie, 1933].

Table II.

Group	October Ca	October P	December Ca	December P	February Ca	February P	March Ca	March P	April Ca	April P
1. Basal	11·55	4·77	9·41	5·88	7·01	8·40	6·29	7·50	6·36	7·59
2. Basal+C.L.O.	11·75	5·19	10·61	5·46	10·52	6·54	10·00	8·17	10·44	7·03
3. Basal+$CaCO_3$	11·68	5·28	10·73	5·12	9·00	5·01	7·75	8·93	7·31	7·12
4. Basal+$CaCO_3$+C.L.O.	11·04	5·27	10·98	5·77	10·91	6·10	10·96	7·99	11·60	7·43

The figures show that serum-Ca fell to extremely low figures in groups I and III, but that C.L.O. retained it at a comparatively normal level.

Discussion.

The effect on growth of lime and cod-liver oil supplements to a Ca-deficient ration here recorded confirm previous work. The results of the blood analysis and balance determinations require further discussion.

Consideration of Table II shows that the average serum-Ca figure for group I had fallen by April to 6·36 mg., an abnormally low figure. That for group III was 7·31 mg., also abnormally low. On the contrary the figure for group II, 10·44 mg., was within the normal range and only slightly lower than the figure for group IV, 11·60 mg.

The logical interpretation of these figures is that the calcium deficiency of the basal ration lowered the serum-Ca of the basal group; that a chalk supplement had little effect in raising it, but that cod-liver oil, either alone or in combination with chalk, restored it to a normal level. It might therefore be assumed that cod-liver oil was more effective than chalk in restoring a normal calcium balance, and

that cod-liver oil alone was almost as effective in this respect as when combined with chalk.

This interpretation of the results of serum-Ca determinations does not correspond with the growth differentiation of the groups or with the findings of the balance experiments. Consideration of Table I shows that a chalk supplement was as effective as cod-liver oil in increasing the positive CaO balance, and that chalk and cod-liver oil in combination were more effective than either alone; results which are in general conformity with the growth differentiation of the four groups.

The indications of the state of calcium metabolism obtained respectively from blood analysis and balance determinations do not agree. The same is true of phosphorus metabolism. Thus in April the figures for blood-inorganic phosphorus were closely similar in all four groups (Table II), whereas there were wide differences in P_2O_5 retention (Table I) at the same period.

While the serum-Ca figures are averages for all five sheep in each group, the balance determinations were done on single sheep and are not therefore strictly comparable. A more reliable guide is to consider the serum-Ca figures for the individual sheep from blood samples drawn at approximately the same dates as those on which their calcium balances were determined (Table III).

Table III.

Group	Index no. of sheep	Av. daily balance g. CaO	Serum-Ca mg./CaO ml.
I. Basal	78	0·08	5·85
II. „ +C.L.O.	27	1·02	10·70
III. „ +Chalk	49	1·02	8·35
IV. „ + „ +C.L.O.	35	1·32	12·00

From Table III it is again apparent that cod-liver oil had a relatively greater effect in raising the level of serum-Ca than in increasing calcium retention. It is also evident that a chalk supplement has a more favourable influence on calcium retention than the serum-Ca figures would suggest.

On the other hand the association of calcium equilibrium with a serum-Ca figure of half the normal value is unexpected. It probably results from minimum Ca excretion owing to the low serum-Ca level. It follows that balance determinations alone might, under certain conditions, give a misleading indication of normality.

From the results recorded in this communication it is evident that growth rate and clinical observation remain the soundest method of determining the effects of supplements added to a calcium-deficient ration. Blood analysis, particularly, cannot be accepted as a satisfactory substitute, until the factors governing serum-Ca level are more perfectly understood. A calcium dietary deficiency undoubtedly lowers the serum-Ca level, at least in sheep, but the effect of different factors in again raising it is not necessarily correlated with their effect on growth or on calcium retention.

Summary.

1. Supplements of chalk or cod-liver oil, fed to sheep on a calcium-deficient ration, resulted in improved growth rate and health, a combined supplement of chalk and cod-liver oil being more effective than either alone.

2. The arrest of growth in the basal, Ca-deficient group was due partly to decreased food consumption and partly to inefficient utilisation of the food consumed.

3. The Ca-deficient ration lowered the serum-Ca, while supplements of chalk or cod-liver oil tended to retain it at a normal level. In this respect cod-liver oil was more effective than chalk.

4. Balance determinations showed that the basal group was in calcium equilibrium; that both chalk and cod-liver oil produced a positive Ca balance; and that their effects were additive.

5. The effect of cod-liver oil in retaining a normal serum-Ca level was much greater than its effect in increasing Ca retention or rate of growth.

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XXIV. THE GLYOXALASE ACTIVITY OF TISSUES.

By MAURICE JOWETT and JUDA HIRSCH QUASTEL.

From the Biochemical Laboratory, Cardiff City Mental Hospital.

(*Received December 21st, 1933.*)

The authors have already investigated [Jowett and Quastel, 1933] the glyoxalase activity of red blood corpuscles. A study has now been made of the glyoxalase activity of tissues with the use of tissue-slices.

An interesting finding, which former workers have not made clear, is that, owing to the high glyoxalase activity of tissues, diffusion of methylglyoxal sets a limit to the activity shown by tissue-slices of the usual thicknesses. The glyoxalase activity is proportional not to the weight of the tissue-slices, but to their surface areas. The observed activity depends on the diffusion coefficient of methylglyoxal in tissues as well as on the intrinsic activity of the cells.

This additional variable makes the designing and interpretation of experiments more difficult. Nevertheless we have been able to obtain a little further information on the part played by methylglyoxal in glycolysis.

Methods.

Rates of glyoxalase action have been measured, as in our former work [Jowett and Quastel, 1933], by the manometric method of Warburg. Methylglyoxal solution, contained in a side-tube, is added to the Ringer's solution after equilibration of the manometers at 37°. Manometric readings are begun 5–10 minutes later.

Because comparatively large weights of tissue are used (usually of the order 5–12 mg., no micro-balance being available), the rate of gas output is high, and measurements cannot be continued for long before changes in the methylglyoxal concentration become appreciable. The high rates however permit fairly accurate measurements of the velocity to be made over a short period.

Methylglyoxal was made by the method of Riley *et al.* [1932], a practically neutral solution being obtained[1]. We have assumed that the gas-output in our anaerobic experiments corresponds to formation of lactic acid. The gas output is completely thermolabile.

The anaerobic glyoxalase activity of tissue-slices.

In Table I are given the results of experiments on the glyoxalase activity of tissue-slices under anaerobic conditions. The only substrate added is methylglyoxal, and the proportion of the gas output due to glycolysis is very small.

Slices of several thicknesses have been employed in each experiment. It is found that the value of $Q_M^{N_2}$ is greater the thinner the slice, and even with the thinnest slices used there is no evidence that $Q_M^{N_2}$ has reached a limiting value.

[1] For the methylglyoxal used in our former work we are indebted to Prof. C. Neuberg.

Table I. *Anaerobic glyoxalase activity of tissue-slices of varying thicknesses.*

At 37°. $N_2+5\ \%\ CO_2$.
Ringer's solution containing 0·025 *M* $NaHCO_3$.
Initial methylglyoxal concentration *M*/100. $v_f=3{\cdot}2$ ml.
Measurements relate to the period about 7–25 minutes after addition of methylglyoxal.
$Q_M^{N_2}$ = mm.³ CO_2 evolved per hr. per mg. dry weight.
$S_M^{N_2}$ = mm.³ CO_2 evolved per hr. per cm.² of surface.

Tissue	Thickness of slice mm.*	$Q_M^{N_2}$	$S_M^{N_2}$
Guinea-pig kidney	0·22	105	240
	0·27	86	235
	0·41	74	(295)
	0·47	51	245
Guinea-pig liver	0·43	29·5	127
	0·43	31·0	133
	0·53	22·8	122
Jensen rat sarcoma	0·27	38·5	(103)
	0·37	34·5	128
	0·37	33·5	123
	0·42	30·5	128
Jensen rat sarcoma	0·32	33	116
	0·37	30	111
	0·45	25·5	116
	0·66	17·5	117
Rat kidney	0·17	110	191
	0·20	96	196
	0·27	70	191
	0·38	61	(235)

* The thicknesses of the slices are calculated by the approximate assumption that the volume of a slice in mm.³ is five times its dry weight in mg.

On the other hand *S*, the rate per unit surface[1], is constant within the experimental error. (The data have not been selected, and some irregularities are evident.)

These findings show that methylglyoxal in appreciable quantity is penetrating only a short distance into the slices. Only a thin outer layer of the slices contributes appreciably to the metabolism; hence the proportionality to the surface area. If sufficiently thin slices were studied, $Q_M^{N_2}$ should rise to a value almost independent of the thickness, and *S* should decrease as slices below the limiting thickness are reached. Our experiments have not been made with slices sufficiently thin to show these limiting phenomena, and consequently we have no direct method of deducing from our data either the maximum value of $Q_M^{N_2}$ or the limiting thickness.

It is clear that our values of $Q_M^{N_2}$, in common with those of former workers, do not measure the glyoxalase activity of tissue cells supplied with a definite concentration of methylglyoxal but give a mean activity averaged over all the cells of the tissue-slice, some of these cells obtaining practically no methylglyoxal.

The values of *S*, the activity per unit surface, are however characteristic for each tissue. *S* will be a function both of the rate at which cells can transform methylglyoxal into lactic acid and of the diffusion coefficient of methylglyoxal. Under certain conditions [Jowett, 1934] the value of *S* is given by the equation

$$S=\sqrt{2ADCo} \qquad \text{.....(1),}$$

[1] *S* is defined in Table I. In calculating surface both sides of the slice are counted, and the area due to the edges has been neglected.

where A is the amount of methylglyoxal consumed per unit volume of tissue adequately supplied with it[1], D is the diffusion coefficient of methylglyoxal in the tissue, and Co is the concentration of methylglyoxal in the outer medium.

The diffusion coefficient, D, may not change much from one tissue to another, and variations in S may be chiefly caused by differences in A, which measures the capacity of tissues to transform methylglyoxal when supplied with it. From the data in Table II it seems likely that A, and therefore the maximum $Q_M^{N_2}$, is higher for kidney than for liver and tumour tissue. The figures in Table II each relate to tissue from a different animal and show that S for any one tissue is fairly constant from one individual animal to another.

Table II. *Anaerobic glyoxalase activity of tissues.*

Conditions as in Table I.

Tissue	$S_M^{N_2}$
Guinea-pig kidney	240, 240, 232, 271
Guinea-pig liver	127, 130
Jensen rat sarcoma	126, 115
Rat kidney	193, 175

In Table III are given the highest values of $Q_M^{N_2}$ which we have observed for various tissues. In each case, the figures provide a lower limit to the maximum value of $Q_M^{N_2}$. The values found by other workers are given for comparison. Conclusions as to the relative activity of glyoxalase in the tissues

Table III. *Anaerobic glyoxalase activity of tissue-slices.*

Conditions as in Table I.

Tissue	Highest observed value of $Q_M^{N_2}$
Rat spleen	50
,, testis	60
,, brain	46
,, sarcoma (Jensen)	38
,, kidney	110
Guinea-pig brain	107
,, testis	54
,, kidney	105
,, liver	40
,, lung	40

Measurements by other authors.

Tissue	$Q_M^{N_2}$	Author
Rat liver	24	Warburg *et al.* [1924]
Flexner rat carcinoma	29	,,
Brain (rat?)	25–50	Meyerhof [1925]
Liver (rat?)	30	,,
Diaphragm (rat?)	15	,,
Rat liver	55	Rosenthal [1930]

cannot be drawn from these data. The thickness of the slices varies considerably, and furthermore the limiting thickness for maximum activity must also vary. We cannot yet estimate how far we are from the maximum values. For human red blood corpuscles we found [Jowett and Quastel, 1933] values of $Q_M^{N_2}$ about 55–75, which are presumably maximum. Other cells may be expected to show higher limiting values, and values up to 200 are quite conceivable.

[1] Regions partially but not adequately supplied are neglected.

The observed anaerobic glyoxalase activity of most tissues, using tissue-slices in 0·01 *M* methylglyoxal, falls off fairly rapidly as time passes. This is illustrated in Fig. 1, where the rates of gas output are plotted against time, taking the initial rate in each case arbitrarily to be 100. The first measurement of rate relates to a time about 10 minutes after methylglyoxal is added. While the velocity-time curves are similar for duplicate slices of any one tissue from the same individual animal, there is much variation from one animal of the same species to another. The variability from one tissue to another shows that the effect can only be due in a minor degree to the diminution in substrate concentration with time.

The behaviour of red blood corpuscles is shown in Fig. 2. The rate in 0·01 *M* methylglyoxal falls off slowly. In 0·02 *M* methylglyoxal the decrease in rate is greater. The decrease in 0·005 *M* methylglyoxal is chiefly due to the low substrate concentrations which are soon reached as the methylglyoxal is consumed. The experiment shows that, in a case where diffusion should be adequate to saturate the enzyme system with methylglyoxal, the maximum value of $Q_M^{N_2}$ is reached at a low methylglyoxal concentration. Raising the methylglyoxal concentration evidently has a toxic effect on the enzyme system, for the initial value of $Q_M^{N_2}$ is lower in the higher concentrations, and the rate of decrease in $Q_M^{N_2}$ is greater.

That tissue-slices are damaged by methylglyoxal is suggested by their change in appearance, on which Kisch [1932] has already remarked. Tissue-slices usually become tougher and firmer, and frequently swollen and opaque, after exposure to 0·01 *M* methylglyoxal in Ringer's solution. Slices of brain become fragile to handle. Loss of pigment, which normally takes place with slices of spleen and liver, is prevented. The changes in appearance are more rapid in higher methylglyoxal concentrations; in concentrations below 0·01 *M* they are less marked.

From these changes, it would not be surprising if the diffusion coefficient of methylglyoxal in tissues altered as time passed. The behaviour of rat testis, shown in Fig. 3, may be due to such changes. The observed glyoxalase activity goes through a maximum at the higher concentrations, which may be due to an initial lowering in the ability of methylglyoxal to diffuse, which then disappears as the membranes are further damaged. The diffusion of sodium lactate through muscle shows analogous changes [Eggleton *et al.*, 1928].

Fig. 4 illustrates the behaviour of guinea-pig liver. In this experiment 0·01 *M* methylglyoxal apparently had little toxic effect on the enzyme system, while higher concentrations exerted progressive inhibitory action. Both Figs. 3 and 4 illustrate the tendency for high methylglyoxal concentrations to produce higher apparent glyoxalase activity. We consider that this effect is due to a thicker layer of tissue being supplied with substrate.

It is only in the case of red blood corpuscles that we can safely attribute the effects of raising the methylglyoxal concentration entirely to toxic action. With tissue-slices we have as additional factors: (1) the better supply of methylglyoxal to the tissue, and (2) possible changes in the diffusion coefficient of methylglyoxal.

In carrying out experiments on the effect of varying conditions on the observed glyoxalase activity of tissue-slices, two methods have been used:

(1) The measurement of activity per unit area of surface (*S*).

(2) The use of tissue-slices of equal thickness, activity then being stated per unit weight (in terms of $Q_M^{N_2}$). By choosing slices carefully, and then cutting each slice into several smaller ones, which are distributed among the several manometric vessels, it is possible to obtain fairly reproducible behaviour, with

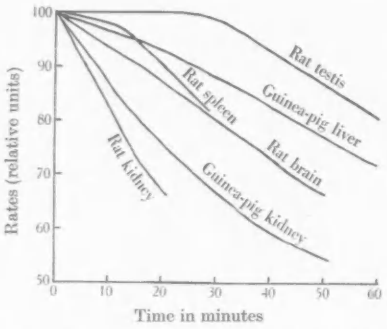

Fig. 1. Anaerobic glyoxalase activity of tissue-slices as a function of time.

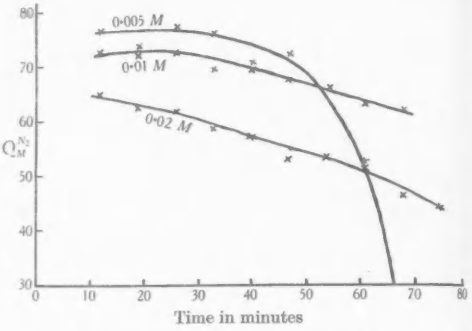

Fig. 2. Glyoxalase activity of human erythrocytes, at several methylglyoxal concentrations.

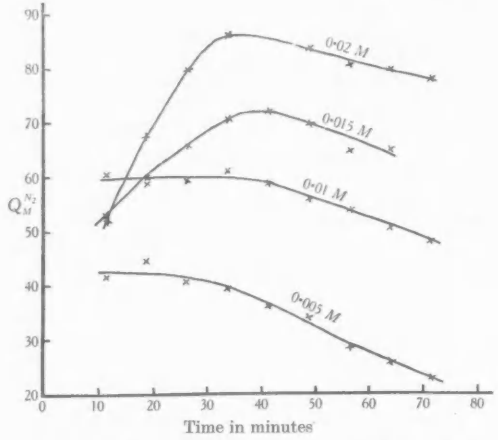

Fig. 3. Glyoxalase activity of rat testis at various methylglyoxal concentrations.

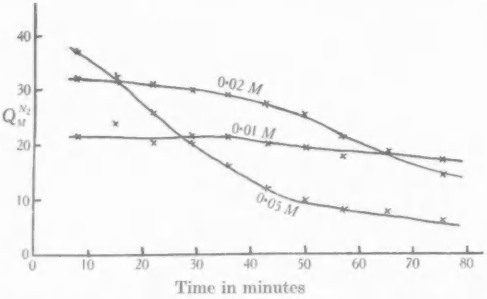

Fig. 4. Glyoxalase activity of guinea-pig liver at various methylglyoxal concentrations.

occasional irregularities. This method, which was used in obtaining the data of Figs. 1, 3 and 4, is applicable in many cases where rectangular slices of reasonable size—for measurement of area—are hard to obtain.

From what has already been said, it should be clear that changes in the observed activity do not necessarily correspond with equal changes in the intrinsic glyoxalase activity of tissues. An additional point will be noted here. If the intrinsic activity falls, the methylglyoxal will penetrate further into the slice (no change in diffusion coefficient being supposed to take place), and as a result the observed change in activity will not be so great as the change in intrinsic activity. When equation (1) holds good, S will change proportionally to the square root of A, if D and Co are constant. The observed values of $Q_M^{N_2}$ will similarly change proportionally to the square root of the maximum $Q_M^{N_2}$, provided that the slices are incompletely saturated with substrate.

According to equation (1) S should vary as the square root of the concentration of methylglyoxal. At very low concentrations of methylglyoxal, according to Jowett [1934], S should vary instead proportionally to the concentration. That is, S varies as c^n, where n changes from 1 at low values of c to 0·5 at high values. Toxic effects are not considered. Measurements have been made of the values of S for guinea-pig kidney at a number of methylglyoxal concentrations; the data, shown in Fig. 5, refer to two different experiments. They appear to fit the linear equation best in dilute solutions and the square-root relation better in stronger solutions. The values of S are however not very suitable for testing the equations. In dilute solutions the substrate concentration is falling off rapidly with time, and in the stronger solutions toxic effects enter.

The influence of glucose on glyoxalase action.

If methylglyoxal is an intermediary in glycolysis, when methylglyoxal and glucose are both presented to a tissue competition between the two substances should occur. More strictly, the competition would be between added methylglyoxal and that produced from glucose. Addition of glucose should not increase the rate of production of lactic acid by a tissue saturated with added methylglyoxal. On the other hand, if glycolysis and glyoxalase action are quite independent, the rates of these processes may proceed independently and additively.

Table IV. *Anaerobic production of lactic acid from glucose and methylglyoxal by tissue-slices.*

Conditions as in Table I. The values relate to a period about 6–20 minutes after methylglyoxal is added. Where glucose is added, its concentration is 0·2 %, and it is present in the Ringer's solution before raising the temperature to 37°.

Tissue	No. of experiments	Mean values of $Q_M^{N_2}$: Substrate absent	Glucose	Methylglyoxal	Methylglyoxal + glucose
Rat spleen	2	2·0	9·5	43	46
Rat testis	4	0·5	8	41·5	40
Rat brain*	4	1·5	16	42·5	58
Guinea-pig testis	2	4·5	11	44	48
Guinea-pig kidney	2	—	8	99·5	98
Jensen rat sarcoma*	2	2·5	27	34	43·5

* The measurements of $Q_M^{N_2}$ in the absence of substrate and in presence of glucose were made in these cases before methylglyoxal was added to obtain the other values for $Q_M^{N_2}$. The effect is to exaggerate relatively the former figures.

Experimentally the difficulty is to saturate tissue-slices with methylglyoxal, and in the experiments given in Table IV we do not know how far this saturation is attained. Competition, if it is taking place at all, may only take place in a small portion of the tissue-slice, the central portions being supplied only with glucose.

The data suggest that some competition is taking place, except in the case of rat brain, and they therefore appear to support to some extent the view that methylglyoxal is an intermediary in glycolysis. Other interpretations are not excluded. We consider that further experiments on this subject should be restricted to tissues, such as leucocytes, which can be adequately supplied with methylglyoxal.

The effect of oxygen on glyoxalase activity.

The glyoxalase activity of human erythrocytes is less in oxygen than anaerobically [Jowett and Quastel, 1933]. With regard to tissue-slices, Meyerhof [1925] has stated that oxygen has no effect on the glyoxalase activity of brain and attributes the lower apparent activity of diaphragm and liver in oxygen to depression of glycolysis.

Respiration and glycolysis are complicating factors in experiments with tissue-slices. By omitting glucose from our Ringer's solution we consider we have avoided appreciable glycolysis in our experiments (except possibly with liver).

As regards respiration, we have adopted, in a first series of experiments, the assumption that respiration proceeds unchanged in presence of methylglyoxal and have corrected the observed manometric changes in oxygen on this basis, employing either measurements of our own for the manometric correction due to respiration, or data such as those of Dickens and Greville [1933].

We have found that the oxygen uptake of slices of guinea-pig liver and kidney is not affected immediately by 0·01 *M* methylglyoxal; appreciable inhibition develops in 30–60 minutes. We have therefore some evidence that Q_{O_2} is unaffected by methylglyoxal. That the R.Q. is unaffected has been assumed without direct experimental evidence.

The observed percentage effects of oxygen on the rate of glyoxalase action of tissue-slices are shown in Table V, the effects before and after correction for

Table V. *Effect of oxygen on observed glyoxalase activity of tissue-slices.*

Conditions as in Table I, except that activity is calculated per unit weight of tissue. Measurements relate to a period 6–20 minutes after addition of methylglyoxal. Rates in $O_2 + 5\ \%\ CO_2$ compared with those in $N_2 + 5\ \%\ CO_2$.

Tissue	No. of experiments	Mean percentage effect of oxygen	
		Uncorrected	Corrected
Guinea-pig liver	5	−18	− 9
,, kidney	4	−16	− 8
Rat testis	4	+ 4	+ 9
,, brain	3	+ 9	+11
,, spleen	3	−24	−14
,, kidney	1	−25	−14
Jensen rat sarcoma	2	−11	0

respiration being given. Evidently the initial effect of oxygen on glyoxalase activity is not large. As time passes, the apparent inhibitory effect of oxygen increases. This is shown in Figs. 6 and 7, in which are given the rates in oxygen (uncorrected for respiration) relative to those in nitrogen as a function of time.

The initial ratio is arbitrarily taken as 100 in each case. In every case the ratio falls off as time passes[1]. If an inhibitory effect of oxygen is not present at first (Table V), it appears to develop as time passes. It is interesting to note that

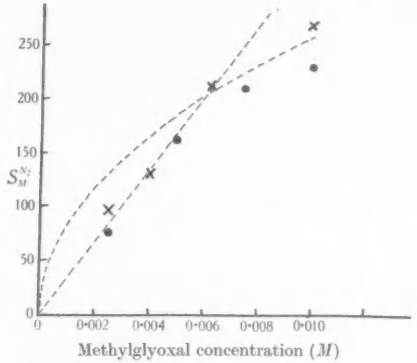

Fig. 5. Glyoxalase activity of guinea-pig kidney per unit surface.

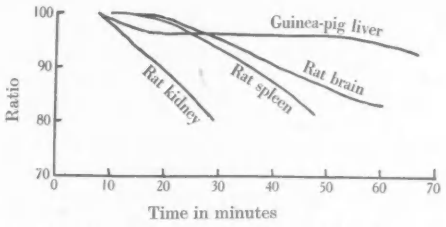

Fig. 6. Ratio of aerobic to anaerobic glyoxalase activity for various tissues.

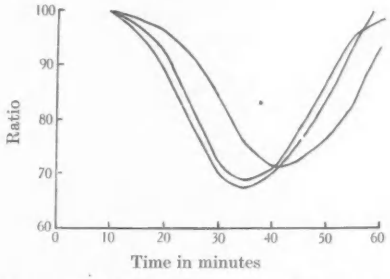

Fig. 7. Rat testis. Ratio of aerobic glyoxalase activity to anaerobic glyoxalase activity.

rat testis shows anomalous behaviour (Fig. 7), the ratio passing through a minimum and then rising again.

In a second series of experiments we have measured aerobic glyoxalase action by the method of Negelein [1925], in which the decrease of bicarbonate concen-

[1] Data for Jensen rat sarcoma are not available.

tration during the experimental period is a measure of acid production. In this series the activity per unit surface of the tissue-slices has been measured. The data obtained are given in Table VI. In these experiments the p_H and bicarbonate concentration change considerably, and hence in each experiment the sizes of slices have been chosen so that the total glyoxalase action in the nitrogen-containing vessel is nearly equal to that in the oxygen-containing vessel.

Table VI. *Anaerobic and aerobic glyoxalase action of tissue-slices.*

$M/100$ methylglyoxal. N_2 or O_2 containing 1·5 % CO_2.
3·2 ml. Ringer's solution containing 0·0073 M $NaHCO_3$.

Tissue	Experimental period mins.	$S_M^{N_2}$	$S_M^{O_2}$	Percentage effect of oxygen
Guinea-pig kidney	49	168	132	−21
,, liver	50	84	72	−14
,, ,,	34	80	66	−17
,, ,,	60	76	50	−34
,, ,,	50	94	69	−26
,, ,,	46	91	70	−23
,, ,,	66	80	62	−23
Cat brain*	37	—	—	−20
,, *	30	—	—	−17

* In the case of cat brain the activity per unit weight was measured.

The experiments show a quite definite inhibitory effect of oxygen on the acid production. Since lactate is being produced by glyoxalase action, in experiments of any length some lactate will be removed by oxidation. We consider, however, that the effect of lactate oxidation on our results will be small.

The real action of oxygen on glyoxalase activity of cells will, according to equation (I), be nearly twice the effect observed on the activity of tissue-slices[1]. Accordingly, the effect of oxygen on the activity of cells may be greater than on that of erythrocytes, for which it amounts to about 20 %.

In considering the effect of oxygen on glyoxalase activity in relation to its possible bearing on glycolysis, it must be remembered that the effect of oxygen may be a function of the methylglyoxal concentration. As methylglyoxal combines with glutathione, the coenzyme of glyoxalase [*cf.* Jowett and Quastel, 1933], a high methylglyoxal concentration may partially protect glutathione from oxidation, and the effect of oxygen on glyoxalase activity may therefore be less at high methylglyoxal concentrations than at low ones. This expectation appears to be realised in the case of human erythrocytes (Table VII).

Table VII. *Glyoxalase activity of human erythrocytes.*

5 % CO_2. $NaHCO_3$ 0·025 M. 0·2 % glucose present. Cells well washed with saline.

	Methylglyoxal 0·02 M			Methylglyoxal 0·005 M		
Exp. No.	$Q_M^{N_2}$	$Q_M^{O_2}$	$Q_M^{O_2}/Q_M^{N_2}$	$Q_M^{N_2}$	$Q_M^{O_2}$	$Q_M^{O_2}/Q_M^{N_2}$
1	62·5	55·5	0·88	67·5	56	0·83
2	63·5	55	0·86	75	58·5	0·78
3	46	44·5	0·97	59·5	46·5	0·78
		Mean	0·90			0·80

If the greater inhibitory effect of oxygen on glyoxalase activity is a general phenomenon at low methylglyoxal concentrations, the supposition that methyl-

[1] Because A, or the maximum $Q_M^{N_2}$, varies as S^2.

glyoxal is an intermediary in glycolysis goes some way to explain the Pasteur phenomenon. Methylglyoxal as an intermediary will be present in tissues only in low concentrations, or glycolysis would proceed much faster than it does.

It may be remarked that the Pasteur phenomenon, *i.e.* the diminution of glycolysis in oxygen, is definitely in need of explanation. The view advanced by Meyerhof and Warburg, that in oxygen tissues can resynthesise lactic acid to carbohydrate, has so far been substantiated only for one mammalian tissue, liver [Cori and Cori, 1929]. Yet liver is the one tissue in which it is doubtful, from the data of Dickens and Greville [1933], whether glycolysis is affected by oxygen.

Lipmann [1933] has put forward the interesting suggestion that the glycolytic ferment may be reversibly oxidised, the oxidised form being inactive[1]. The suggestion rests principally on experiments with dyestuffs which are considered to act solely as oxygen carriers. It is to be remembered, however, in considering Lipmann's experiments that a number of dyestuffs are toxic to ferments [*cf.* Yabusoe, 1925; Quastel, 1932], and it is possible that this toxicity is lost on reduction of the dyestuffs to their leuco-forms.

Glyoxalase activity in relation to glycolysis.

Experiments on muscle extracts have led Embden *et al.* [1933] to propose, and Meyerhof and Kiessling [1933] to support, a mechanism for glycolysis in which methylglyoxal has no place. Their work cannot however permit us to ignore the evidence that methylglyoxal is, in some tissues at least, a probable precursor of lactic acid. Many facts support or are in accordance with such a view.

(1) Tissues treated so as to inhibit glyoxalase activity produce methylglyoxal from hexosediphosphate [Neuberg and Simon, 1933] and from glyceraldehyde-phosphoric acid [Barrenscheen and Beneschovsky, 1933].

(2) All tissues investigated, with the exception of pancreas, can transform methylglyoxal into lactic acid.

(3) The kinetics of glyoxalase action satisfy the conditions required for participation in glycolysis.

(*a*) The rate of transformation of methylglyoxal into lactic acid by tissues is high and should proceed as rapidly as glycolysis at quite low methylglyoxal concentrations. The best evidence for this is provided by the present work. It had previously been in doubt whether the glyoxalase activity of tumour tissue was sufficiently high.

(*b*) The activity of glyoxalase extends over a range of p_H similar to or greater than that of the glycolytic enzyme-system [Kuhn and Heckscher, 1926; Lohmann, 1932].

(*c*) Glyoxalase action is only inhibited by substances which also affect glycolysis to an equal or greater extent. The actions of fluoride [Lohmann, 1933], iodoacetic acid [Dudley, 1931; Lohmann, 1933], and narcotics [Meyerhof, 1925], may be mentioned in this connection. Pancreatic extracts affect both enzyme systems [Harrison and Mellanby, 1930; Giršavičius, 1933] but quantitative comparisons have not been made.

(*d*) It also appears, from the present work, that the effect of oxygen on glycolysis is paralleled to some extent by its effect on glyoxalase action.

It must be admitted that the methylglyoxal theory meets with certain difficulties. Lohmann has shown [1932] that muscle extracts possessing no

[1] This suggestion is formally similar to the view already expressed by the authors [1933] that a partial explanation of the Pasteur phenomenon may be found in the reversible oxidation of glutathione—the co-enzyme of glyoxalase.

glyoxalase activity can break down glycogen to lactic acid. This proves that at least one tissue—or rather one tissue extract—has a mechanism of glycolysis not involving methylglyoxal. Many facts however favour the view that different tissues may have glycolytic mechanisms differing in detail, and even that one tissue may have several mechanisms for glycolysis (*cf.* Ashford, 1933). It can be suggested that several paths from carbohydrate to lactic acid may exist in tissues, and that methylglyoxal is not an intermediary in all of them.

The lactic acid produced by glyoxalase action is also apparently not stereochemically identical with that produced by glycolysis. It is not however certain that glycolysis produces only the *d*-form. It is, alternatively, conceivable that the glyoxalase action of undamaged cells at low methylglyoxal concentrations may produce only the *d*-form, for the variability in the rotations observed suggests that toxic effects may be playing a part.

SUMMARY.

1. An investigation of the glyoxalase activity of tissue-slices shows that diffusion of methylglyoxal into the slices limits the rate of reaction. Glyoxalase action is proportional to the surface areas of tissue-slices, not to their weights. The glyoxalase activity of tissues is greater than has been hitherto supposed.
2. It is possible that methylglyoxal and glucose compete as substrates.
3. Oxygen partially inhibits glyoxalase activity in most tissues.
4. Glyoxalase activity is discussed in relation to glycolysis.

The authors wish to thank Prof. W. C. M. Lewis for the loan of manometric apparatus, and Dr G. D. Greville for the gift of tumour-bearing rats.

One of the authors (M. J.) is indebted to the Medical Research Council for a whole-time grant.

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XXV. THE EFFECT OF PYOCYANINE ON THE RESPIRATION OF SOME NORMAL TISSUES AND TUMOURS[1].

By ERNST A. H. FRIEDHEIM.

(*Received December 23rd, 1933.*)

WARBURG's theory states that the tumour cell suffers from a relative insufficiency of respiratory enzyme, so that even under aerobic conditions the balance of the Pasteur-Meyerhof reaction shows an excess of lactic acid. This suggests that the lack of enzyme might be compensated by the addition of an accessory catalyst. This working hypothesis gains support from the evidence that a similar mechanism actually occurs in nature [Friedheim, 1931]. The *Bacillus pyocyaneus* contains, besides cytochrome, a respiratory ferment similar to that found in higher animals in that it is sensitive to CO and KCN. However, even with free access of molecular oxygen this respiratory enzyme proves insufficient in the absence of assimilable extraneous substances; the oxygen consumption is minimum, the respiratory quotient is almost zero and the oxidation incomplete. Corresponding to those conditions described by Warburg as being characteristic of the tumour cell, *B. pyocyaneus* lives almost as an anaerobic organism even in the presence of air. Under certain conditions it produces the blue pigment, pyocyanine, which, owing to the reversibility of its oxidation and reduction, acts as an accessory oxidation catalyst. In its presence oxygen consumption is increased as much as twenty-four fold and the R.Q. approximates to unity.

It has previously been shown [Friedheim, 1931] that the stimulating effect of pyocyanine on respiration is in no way species-specific but is also to be observed in the red blood corpuscles of rabbits. In the following the effect of pyocyanine on the respiration of some normal and tumour tissues will be described.

EXPERIMENTAL.

Procedure. The Barcroft-Warburg manometric method was employed. Tissue sections 0·2 mm. thick were put with 2 ml. of Ringer's solution into the main chamber of a 10 ml. respiration vessel. For the absorption of CO_2 the inner vessel contained 0·2 ml. of 6 % NaOH, the accessory bulb 0·2 ml. $M/50$ solution of recrystallised pyocyanine. The thermostat was kept at 37·5°. The manometer was shaken at the rate of fifty complete oscillations per minute. The oxygen consumption in Ringer's solution was measured for a first period varying from 30 to 60 minutes. The pyocyanine was then tipped from the accessory vessel into the main chambers, and the observations were continued during a second equal period. This method suffers from the unphysiological condition that the partial pressure of CO_2 is held at approximately zero; but against this drawback stands the great advantage of extraordinary simplicity and the identity of the tissue fragment in the experiment and the controls.

[1] This work has been carried out in the department of Prof. Leonor Michaelis at the Rockefeller Institute for Medical Research, New York. I wish to express my indebtedness and gratitude to Prof. S. Flexner and to Prof. L. Michaelis for the hospitality and the facilities they kindly extended to me.

Tissue sections which were introduced into the previously warmed respiration vessels within a period of less than four minutes after the death of the animal showed the oxygen consumption to be strictly proportional to the time for a period of at least 100 minutes. The deviations to be expected with exclusion of bacterial growth are in the direction of a decrease in respiration and cannot exaggerate any observed increase in respiration. The Ringer's solution contained 0·0025 *M* bicarbonate. In the experiments where sugar was added, a concentration of 0·2 % glucose was used. In those in which phosphate-Ringer was employed, the phosphate concentration was 0·01 *M* and the p_H 7·4.

Results. As can be seen from Tables I–III, the respiration of rat kidney in carbonate-Ringer is either uninfluenced or slightly impeded by pyocyanine; whereas the respiration of testicular tissue, and more so that of tumours, is very

Table I. *Respiration of rat kidney.*

Corrected manometric values for mm.^{3} O_2 absorbed obtained in two periods of equal duration, the first without, and the second with, pyocyanine.

Duration of exp.	30–60 mins.
Thickness of section	0·2 mm.
Glucose concentration	0·2 %
Atmosphere	O_2
Temperature	37·5°
Final pyocyanine concentration	*M*/500
Absorption of CO_2 by 6 % NaOH	

	In carbonate-Ringer 2·5/1000 *M*			In phosphate-Ringer (1/100 *M*) p_H 7·4		
Exp. No.	Without* pyocyanine	With pyocyanine	Difference %	Without pyocyanine	With pyocyanine	Difference %
1	26·0	25·6	− 1·5	18·0	22·1	+22·7
2	19	16·5	−13·15	22	29	+31·8
3	32	26	−12·5	31	31·8	+ 2·5
4	18·5	18·0	− 2·7	25	36	+ 3·6
5	35	31	−11·4	35	37·5	+ 7·1
6	35	37	+ 5·7	50	53·0	+ 6·0
7	30·4	30·7	+ 1·9	54·0	60·2	+11·5
		Mean difference	−0·5 %		Mean difference	+16·8 %

* Mean value of Q_{O_2}, 14·2.

Table II. *Respiration of testicular tissue (rat).*

Corrected manometric values for mm.^{3} O_2 absorbed obtained in two periods of equal duration, the first without, the second with, pyocyanine.

Testicular tubuli carefully dissociated to form only one layer.

Ringer solution	$NaHCO_3$ 2·5/1000 *M*
Duration of exp.	30–60 mins.
Glucose	0·2 %
Final pyocyanine concentration	*M*/500
Atmosphere	O_2
Temperature	37·6°
Absorption of CO_2 by 6 % NaOH	

Experiment No.	Without* pyocyanine	With pyocyanine	Difference %
1	59	67	+30·5
2	43	49	+13·9
3	53	58	+ 9·4
4	59	68	+15·2
5	22	30	+30
		Mean difference	+19·8 %

* Mean value of Q_{O_2}, 9·4.

Table III. *Respiration of tumour tissue and rat testicle in Ringer solution 2·5/1000* M $NaHCO_3$.

(Conditions the same as in Table I).

Tissue	Experiment No.	With 0·2 % glucose: Without pyocyanine	With 0·2 % glucose: With pyocyanine	With 0·2 % glucose: Difference %	Without glucose: Without pyocyanine	Without glucose: With pyocyanine	Without glucose: Difference %
Mouse sarcoma S 37	1	23	32·7	+42	35	23·5	−25·7
	2	20	32	+60	39	33	−15·3
	3	39	54	+38·5	25	28	+12
	4	23	62	+170	20	20	0·0
	5	23	31·3	+36	27	24·1	−11
	6	57	78	+36·9			
	7	28	34	+23·5			
	8	29	61·6	+105	26	30·3	+16·5
		Mean values		+63·8			−4 %
Mouse sarcoma S 159	1	16·4	24	+46·3	25	27	+8
	2	19	33	+73·7	23	27	+17·4
	3	18	24	+33·7	18	16	−11
	4	29	39	+37·9			
	5	18	22	+22·2	23	23	0·0
	6	22	32	+45·4	26	28	+7·7
		Mean values		+43·2			+2·4
Human breast carcinoma	1	9	23	+155	7	8	+12·5
	2	22·5	29·6	+31·8	18	18	0·0
	3	10	24	+140			
Spindle-cell sarcoma of humerus	1	23·5	42	+78·8	14	14	0·0
Neuro-blastoma of retina	1	12	27	+125	20	37	+75
Rat testicle (mm.^{3} O_2/hr./mg.)	1	9·02	11·65	+29·7	7·4	7·4	0·0
	2	9·72	12·31	+26·6	7·2	7·2	0·0

considerably enhanced. It is worthy of note that the presence of glucose is essential for the increase of respiration observed with pyocyanine. This is contrary to the findings in the case of bacteria, where the catalytic effect of pyocyanine is exercised on the substance of the cell itself only in the absence of extraneous assimilable substances.

The relative toxicity of pyocyanine and methylene blue.

Harrop and Barron [1928] were the first to show that the respiration of living cells can be increased by the catalytic action of reversible dyestuffs such as methylene blue. These authors found, in accordance with the theory, that the accessory catalyst could only enhance the respiration in cases where there is aerobic glycolysis, *i.e.* where the respiration is insufficient to bring about the disappearance of all lactic acid in the Pasteur-Meyerhof reaction. Our experiments confirm in principle those of Barron [1930], as they show that kidney, a tissue without aerobic glycolysis, is uninfluenced by pyocyanine in its respiration, or if anything, slightly hindered; whereas in tumours with positive aerobic glycolysis the respiration is considerably increased. In accordance with their theoretical considerations the above-mentioned authors expected to find that in the case of testicular tissue, which exhibits aerobic glycolysis, the respiration would be increased on the addition of methylene blue. However, methylene blue

failed to bring about this effect, which, as can be seen from Table II, was brought about by pyocyanine. As the potential range of the two dyestuffs is of the same order of magnitude (E_0 pyocyanine $= -34$ mv. E_0 methylene blue $= +11$ mv. at p_H 7·0), the cause of the dissimilarity in their actions is to be sought in a difference in toxicity. In the case of testicular tissue, the catalytic action seems to be almost exactly compensated by the toxic action. Pyocyanine, probably owing to its small affinity for proteins and high affinity for lipoids, is much less toxic than methylene blue. In the previous experiment with rabbit erythrocytes, pyocyanine caused an increase in respiration of 1250 % (duration of experiment, one hour), a figure which is just above the best values reported by Harrop and Barron [1928] and Michaelis and Salomon [1930] with methylene blue.

In normal rat kidney, which shows practically no glycolysis, the toxicity cannot be masked by any catalytic action. In spite of this fact the average inhibition of respiration brought about by pyocyanine is only 5·8 %, while with methylene blue, according to Harrop and Barron, this amounts to 13·8 %. It is to be emphasised that our experiments were carried out with carbonate-Ringer, while those of Harrop and Barron were done with phosphate-Ringer.

Influence of phosphate.

In opposition to Dixon and Elliott [1929], Warburg [1931] has strongly emphasised the unphysiological action of phosphates. Using phosphate solution, Dixon found that the inhibition of tissue respiration by cyanide was incomplete; in other words, the phosphate solution caused a relative increase in the respiration. An effect of phosphate in the same sense was apparently observed by Barron, since he reports that in phosphate solution the catalytic action of methylene blue on the respiration of rabbit erythrocytes is increased. Table I shows that in normal rabbit kidney in the presence of phosphates pyocyanine causes a considerable increase in respiration which does not occur in carbonate-Ringer.

In Barron's [1930] experiments methylene blue inhibited the respiration of rat kidney even in a phosphate-Ringer solution, which in itself tends to increase the catalytic action. On the other hand, under the same conditions pyocyanine induces an increase of 16·8 %. Hence the difference in toxicity of the two dyestuffs is readily demonstrated.

Recently Dickens and Šimer [1930] have used phosphate-Ringer for determining the R.Q. in tissue sections. In control experiments these authors have compared the standard values of the respiration (Q_{O_2}) in carbonate- and in phosphate-Ringer. These authors, having obtained results which coincide within the limits of experimental error, feel justified in assuming the conditions in phosphate-Ringer to be physiological and in according an important rôle to the values of the R.Q. determined in such solutions. In view of the above experiments it seems to us that the comparability of the standard values of respiration in carbonate- and phosphate-Ringer offers no guarantee that the tissues in phosphate-Ringer are under physiological conditions. The great increase in respiration caused by pyocyanine in phosphate- but not in carbonate-Ringer in normal rat kidney seems strongly to indicate that in phosphate solution the oxidation mechanism of the cell is fundamentally different.

The mode of action of pyocyanine.

Increase in depth of respiring layer of tissue sections. Warburg [1923] has drawn attention to the fact that the metabolic exchange is restricted to a more or less thick cortical layer, while the central portion remains inactive. The thickness of

the layer where molecular oxygen is available can be calculated according to Warburg as

$$d = \sqrt{8c_0 \frac{D}{A}}$$

where d = thickness of layer where oxygen is available,
c_0 = oxygen concentration at the limits between section and solution,
D = diffusion constant of oxygen for tissue substance,
A = the oxygen consumption of the tissue per unit volume and time.

If we regard D as the sum of the diffusion constants of all substances which can act with respect to the tissue as hydrogen acceptors, then D will be increased on addition of pyocyanine by a value equivalent to the diffusion constant of the dye solution in the tissue. As pyocyanine, and to a lesser extent methylene blue, are extremely lipoid-soluble, they have a tendency to accumulate in the tissues to a concentration exceeding the value which would be reached by simple diffusion. Thus there results a considerable increase in D with a corresponding increase in d according to the formula. So far this would only signify an increase in the depth of the tissue layer taking an active part in oxidation metabolism but have no bearing on the measurable oxygen consumption. But as this process goes on, the reduced form of the dye tends to accumulate in the depth of the tissue and a diffusion gradient of the leuco-compound will be established from within the tissue towards the surface (and perhaps oxidisable substances produced by the dehydrogenation of the tissue). Here the leuco-compound, diffusing out of the tissue will immediately be oxidised by the molecular oxygen and thus the oxygen consumption actually increased. It follows that in tissue sections which exceed the thickness in which molecular oxygen would be available, the addition of reducible dyestuffs induces an increase in respiration which is purely mechanical without involvement of the fundamental chemistry of the cell.

Table IV. *Influence of thickness of section on the respiration of rat kidney.*

Ringer solution — 2·5/1000 M $NaHCO_3$
Glucose — 0·2 %
Final pyocyanine concentration — M/500
Atmosphere — air
Absorption of CO_2 by NaOH
Temperature — 37·6°

Experiment No.	Thickness of section 0·2 mm. mm.³ O_2 consumed in 30 mins. Without pyocyanine	With pyocyanine	Difference %	Thickness of section 3·0 mm. mm.³ O_2 consumed in 30 mins. Without pyocyanine	With pyocyanine	Difference %
1	46	44·5	−3·2	108	132	+13·3
2	18	16·5	−8·3	55	80	+45·5

This effect is illustrated by Table IV. As has been shown in Table I, the respiration of rat kidney cut in sections not thicker than the layer accessible to molecular oxygen (0·2 mm. in an atmosphere of oxygen) is not increased on the addition of pyocyanine; in sections 3·0 mm. thick pyocyanine enhances the respiration as much as 45 %.

The catalytic action. As has been shown in the case of bacteria [Friedheim, 1931] in thin sections of tissue the mechanism of the respiration brought about by pyocyanine may be explained as a true catalysis in that the dye is reduced by

the tissue and reoxidised by the molecular oxygen. The significance of the increase in respiration for the cell may be judged by the influence of pyocyanine on the aerobic glycolysis.

Table V. *Aerobic glycolysis of various tumours, expressed as* $Q^{O_2}_{CO_2}$, i.e. *mm.*^{3} CO_2 *driven out of a bicarbonate solution by lactic acid formed per hour per mg., dry weight of tissue.*

Ringer solution	0·025 *M* $NaHCO_3$
Glucose	0·2 %
Atmosphere	5 % CO_2 in O_2
Temperature	37·5°
Final pyocyanine concentration	*M*/500
Thickness of tissue section 0·2 mm.	

Experiment No.	Tumour	$Q^{O_2}_{CO_2}$ Without pyocyanine	$Q^{O_2}_{CO_2}$ With pyocyanine	Difference %	Without pyocyanine I Respiration Q_{O_2}	Without pyocyanine II Anaerobic glycolysis	Without pyocyanine III U[1]
1	Cori rat sarcoma	20·1	13·1	−34·7	12·5	26·1	+ 1·1
2	Walker rat carcinoma 256	11·4	9·0	−21	6·36	22·1	+ 8·4
3	Walker rat sarcoma S 20	18·5	7·88	−87·6	13·3	68·2	+41·6
4	„	40·4	25·9	−35·8	17·94	62·5	+32·5
5	„	8·51	0·0	−100			
6	Mouse sarcoma	13·0	5·46	−58			
7	„	15·3	6·0	−60·8			
8	Mouse sarcoma S 27	19·9	9·76	−50·9			
9	Human breast carcinoma	5·2	3·4	−34·6			
10	„	6·8	4·4	−22·2			
11	Human spindle-cell sarcoma	5·28	3·1	−41·3			
12	Cori rat sarcoma	13·0	13·0	0·0	−13·9	24·3	− 3·5
13	Walker rat sarcoma	23·7	22·2	0·0	−16·25	28·6	− 4·4

Table V shows that in a series of tumours the aerobic glycolysis is actually decreased on the addition of pyocyanine. The two exceptions in Table VII (Exps. 12 and 13) happen to be tumours with a negative U value[1], *i.e.* tumours in which theoretically the respiration would be sufficient to suppress aerobic glycolysis. Attention has recently been drawn by several authors to the existence of tumours which show at the same time high respiration and low glycolysis. As suggested by Warburg the respiration in tumours may be not only quantitatively insufficient to dominate glycolysis, but as in the case of ethyl carbylamine poisoning, by the coupling of these two processes the mechanism of the Pasteur-Meyerhof reaction might be broken.

[1] U stands for the anaerobic glycolysis *minus* twice the value of the respiration. This concept of U was introduced by Warburg as a measure of the efficiency of respiration since two molecules of oxygen are sufficient to cause the disappearance of one molecule of lactic acid during the course of the Pasteur-Meyerhof reaction. Where $U < 1$, the respiration is "sufficient" and there is no aerobic glycolysis.

Also in our positive experiments in which pyocyanine decreases aerobic glycolysis without eliminating it entirely it must be remembered that the measurements were carried out in Ringer's solution, which is liable in itself to damage the Pasteur-Meyerhof reaction.

In view of the difficulties in interpretation it might seem that the biological significance of the increased respiration brought about experimentally by accessory catalysts, especially in its bearing on the question of malignancy, might best be brought out not with surviving tissue sections but with living cells in animal experiment, or in tissue cultures, as suggested by Barron[1]. For such experiments it is important to have a catalyst available which combines the properties of minimum toxicity and maximum respiratory catalysis. Pyocyanine appears to fulfil these requirements.

Summary.

1. Pyocyanine increases the respiration of tissues with aerobic glycolysis such as testicular tissue and a series of animal and human tumours.

2. The increase in respiration is dependent on the presence of glucose.

3. In this action one must differentiate between an apparent increase in respiration, brought about in thick tissue sections through an increase in the depth of the respiring layer, and a true catalysis.

4. While in carbonate-Ringer the respiration of normal rat kidney is not increased by pyocyanine, a considerable increase is brought about in phosphate-Ringer. In the presence of phosphate the oxidation mechanism of the cell seems to be fundamentally altered.

5. The toxicity of pyocyanine is compared with that of methylene blue. Pyocyanine shows minimum toxicity and enormous action in increasing respiration.

We wish here to express our gratitude to Drs Murphy, Landsteiner, Walker and Klemperer for furnishing animal and human tumour material.

[1] These experiments are under way.

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XXVI. NATURAL REVERSIBLE OXIDATION-REDUCTION SYSTEMS AS ACCESSORY CATALYSTS IN RESPIRATION: JUGLON AND LAWSON.

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(*Received December 23rd, 1933.*)

Lawson, the colouring material contained in the leaves of the henna plant (*Lawsonia inermis*), and juglon, the colouring material contained in the husks of walnuts, both form leuco-compounds when exposed to the influence of various reducing agents. The leuco-compounds at once regenerate the pigment in contact with molecular oxygen. Thus they fulfil one of the main conditions which might enable them to play a part in cell-respiration by acting as autoxidisable acceptors of hydrogen.

This paper deals with the effect of juglon and lawson on cell-respiration, the establishment of the oxidation-reduction potentials of both substances and the correlation of the two series of results.

The comparison between the physicochemical and biological properties of the two substances appears all the more attractive in view of the fact that they are isomeric compounds, their formulae being 2-hydroxynaphthoquinone [Tommasi, 1920] for lawson, and 5-hydroxynaphthoquinone for juglon [Bernthsen and Semper, 1885].

A. *Juglon.*

Preparation. Juglon was obtained by extraction of the husks of walnuts by the method of Bernthsen and Semper and also synthetically by oxidation of 1 : 5-dihydroxynaphthalene with chromic-sulphuric acid mixture and subsequent crystallisation from light petroleum. The natural and synthetic products gave identical results in the physicochemical and biological experiments described below.

Potentiometric titration. In order to determine the oxidation-reduction potentials, 10 ml. of $M/1000$ solution in various mixtures of buffer substances were first reduced by hydrogen in the presence of colloidal palladium and subsequently titrated against benzoquinone in a current of purified nitrogen at a temperature of $20° \pm 0{\cdot}1°$. The potentials were read against two polished platinum electrodes, using a saturated half-cell of calomel as standard. The potentials adjusted themselves rapidly and remained stable, and the readings for both electrodes differed by not more than 0·05 mv. during the middle phase of titration.

The potentials thus determined correspond to the formula of Nernst:

$$E = E_0 + \frac{RT}{n} \log \frac{(OX)}{(RED)}.$$

The system juglon-hydrojuglon can therefore be regarded as thermodynamically reversible (see Table I).

Table I. *Juglon.*

$p_H = 5{\cdot}13$ (phosphate) reduced with Pd-H_2 titrated against quinone at $20{\cdot}0° \pm 0{\cdot}05°$.
Potentials calculated according to the formula $E = 0{\cdot}1332 + 0{\cdot}029 \log \frac{\%\text{ oxidation}}{100 - \%\text{ oxidation}}$.
All potentials referred to the standard hydrogen electrode at 20°.

Quinone ml.	Oxidation	Potential		
		Found	Calculated	Discrepancy
0·00	0·0	—	—	—
0·35	15·8	0·1062	0·1121	−0·0059
0·50	22·6	0·1152	0·1177	−0·0025
0·64	28·8	0·1206	0·1218	−0·0012
0·80	36·1	0·1254	0·1260	−0·0006
1·02	45·9	0·1308	0·1311	−0·0003
1·26	56·7	0·1371	0·1366	+0·0005
1·58	71·2	0·1457	0·1446	+0·0011
1·76	79·3	0·1520	0·1501	+0·0019
2·02	91·0	0·1670*	0·1622	+0·0048
2·17	97·6	0·1866*	0·1799	+0·0067
2·22	100·0	0·2672*	—	—

* Potentials drifting.

The determination of n, *the number of electrons which distinguish the reduced from the oxidised form.* According to the chemical constitution of the substance, n must be 2. This statement is corroborated by the oxidation equivalent found by titration, as well as by the slope of the titration curve.

(*a*) Hydrojuglon uses up equimolecular amounts of benzoquinone during its oxidation.

Exp. Juglon mol. wt.: 174.
Standard solution: 43·557 mg. in 50 ml. ethyl alcohol.
1 ml. corresponds to $0{\cdot}5 \cdot 10^{-5}$ *M*.

Benzoquinone mol. wt.: 108.
Titration solution: 44·16 mg. in 100 ml. dist. H_2O.
1 ml. corresponds to $4{\cdot}083 \cdot 10^{-5}$ *M*.

2 ml. of the standard solution of juglon reduced with Pd-H_2 in 8 ml. of acetate buffer solution of $p_H = 4{\cdot}62$, require 2·44 ml. of titrating solution for oxidation in a current of nitrogen (the complete oxidation being indicated by a jump of potential).

$1{\cdot}0 \cdot 10^{-5}$ *M* juglon is equivalent to $\frac{2{\cdot}44 \times 4{\cdot}083}{100} = 0{\cdot}996 \cdot 10^{-5}$ *M* benzoquinone.

(*b*) At acid reactions, the titration curves clearly show a 30 mv. slope, corresponding to $n = 2$. When the p_H reaches 7, the curves become steeper, without reaching however a slope of 60 mv. This irregularity, which points towards a formation of semiquinone or meroquinone, will not be further discussed here, considering the instability of juglon in alkaline media.

The standard potential E_0 (determined in a mixture of equal parts of the oxidised and reduced forms of the substance) is shown in Fig. 1 and Table II as a function of the p_H. According to Clark and Gibbs, the turning-points of the E_0/p_H curve can be interpreted as representing constants of dissociation (see Table V).

B. *Lawson.*

The pure substance was obtained by extraction from henna leaves by the method of Tommasi; the calcium salt was crystallised and the free hydroxyquinone recrystallised from ether. Electrometric titration by means of the method

Table II.

The normal potential of juglon (mixture of equal parts of the oxidised and reduced form) at varying p_H. Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
1·994	Citrate	+0·311	Quinone
3·263	,,	+0·239	K_3FeCy_6
4·746	Acetate	+0·154	Quinone
5·133	Phosphate I and II	+0·133	,,
5·707	,,	+0·103	,,
6·263	,,	+0·071	,,
6·799	,,	+0·041	,,
7·316	,,	+0·023	,,
8·001	,,	0·000	,,
8·389	,,	−0·023	,,
9·401	II Phosphate and NaOH	−0·071	,,
10·785	,,	−0·131	,,
11·41	,,	−0·160	$K_2S_2O_8$
11·929	,,	−0·175	Quinone
12·10	,,	−0·185	$K_2S_2O_8$

Table III. *Lawson.*

$p_H = 2·24$ (citrate + HCl) reduced with Pd-H_2 titrated against quinone at 20·0° ± 0·05°.

Potentials calculated according to the formula $E = 0·2198 + 0·029 \log \frac{\%\text{ oxidation}}{100 - \%\text{ oxidation}}$.

All potentials referred to the standard hydrogen electrode at 20°.

Quinone ml.	Oxidation %	Potential		Discrepancy
		Found	Calculated	
0·00	0·0	—	—	—
0·14	7·2	0·1828*	0·1870	−0·0042
0·24	12·4	0·1929	0·1947	−0·0018
0·44	22·9	0·2036	0·2040	−0·0004
0·66	33·8	0·2107	0·2108	−0·0001
0·86	44·3	0·2162	0·2163	−0·0001
1·08	55·6	0·2222	0·2221	+0·0001
1·34	69·0	0·2295	0·2293	+0·0002
1·56	80·6	0·2379	0·2372	+0·0007
1·73	89·0	0·2470	0·2455	+0·0015
1·88	96·8	0·2692*	0·2615	—
1·91	98·0	0·2912*	0·2682	—
1·94	100·0	0·3500*	—	—

* Potentials drifting.

described in the case of juglon shows that we are dealing again with a true reversible system. All the titration curves show a slope of 30 mv., corresponding to $n = 2$. An example is recorded in Table III. The dependence of the standard potential E_0 is shown in Table IV and Fig. 1, and the values for the dissociation constants which can be derived from the former are shown in Table V.

C. *A comparison between the various constants of juglon and lawson.*

The 2-hydroxynaphthoquinone, lawson. is more acid and more "negative" than juglon. As compared with juglon the acid character of lawson is enhanced, not only in the phenolic hydroxyl of the oxidised form, but also in the hydroxyl of the reduced form. In the case of lawson, the first dissociation constant of the reduced form is so close to the constant of the oxidised form that the

Table IV.

The normal potential of lawson (mixture of equal parts of the oxidised and reduced form) at varying p_H. Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
0·360	*N* HCl + *N*/10 KCl	+0·337	K_3FeCy_6
1·322	Citrate	+0·2757	Quinone
1·423	,,	+0·270	,,
2·245	,,	+0·220	,,
2·460	,,	+0·2087	,,
3·248	,,	+0·1635	,,
3·680	,,	+0·1298	,,
3·881	,,	+0·1205	,,
4·280	,,	+0·0862	,,
4·620	Acetate	+0·061	,,
4·945	,,	+0·032	,,
6·194	Phosphate I and II	−0·071	K_3FeCy_6
6·641	,,	−0·1105	Quinone
7·180	,,	−0·154	,,
7·560	,,	−0·186	K_3FeCy_6
8·718	Veronal	−0·281	Quinone
9·180	,,	−0·318	$K_2S_2O_8$
10·075	II Phosphate and NaOH	−0·3648	Quinone
10·510	,,	−0·395	$K_2S_2O_8$
11·34	,,	−0·428	,,
12·23	,,	−0·461	,,

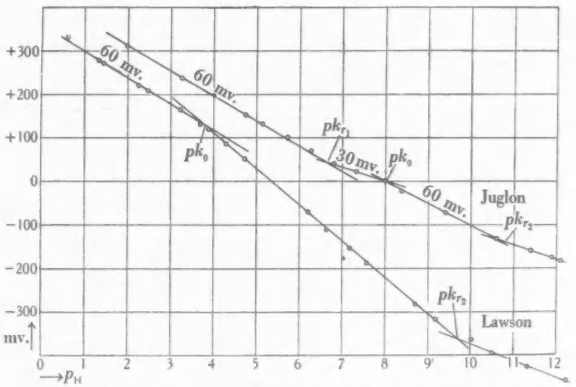

Fig. 1. Normal potentials of juglon and lawson at varied p_H. Potentials referred to the standard hydrogen electrode at 20°.

Table V.

	Juglon	Lawson
Dissociation constant of the oxidised form (of the phenolic hydroxyl)	K 10^{-8}	K $1{\cdot}4.10^{-4}$
1st dissociation constant of the reduced form	$2{\cdot}5.10^{-7}$	About $1{\cdot}4.10^{-4}$
2nd dissociation constant of the reduced form	$2{\cdot}5.10^{-11}$	$2{\cdot}4.10^{-10}$

Temperature: 20°.

two cannot be sharply separated. The flattening effect of the pk_{r_1} combines with the steepening effect of pk_0 to give to the E_0/p_H curve a slope of 84 mv.

Conant and Fieser [1924] have found that the introduction of the hydroxyl group into the quinoid nucleus of α-naphthoquinone lowers the oxidation-reduction potential much more markedly than its introduction into the adjoining benzene ring. Their results support our findings with regard to the increased negativity of lawson as compared with juglon. These authors have determined the oxidation-reduction potentials of the two substances exclusively in strong alcoholic-hydrochloric acid solution. On account of the difference of the solvents the values obtained by them cannot be directly compared with those recorded in this paper.

The relative positions of juglon and lawson in the scale of oxidation-reduction indicators can be seen in Table VI.

Table VI. *Potentials of several oxidation-reduction systems in semi-reduced condition at* $p_H = 7{\cdot}0$.

(Potentials referred to the normal hydrogen electrode.)

Haemoglobin-methaemoglobin	+0·092 ±0·022	Conant und Fieser [1925]
Pigment of *Penicillium phoeniceum*	+0·047	Friedheim [1933, 3]
Juglon	+0·033	
Pigment of *Arion rufus*	+0·025	Friedheim [1933, 1]
Hallachrome	+0·022	Friedheim [1933, 2]
Methylene blue	+0·011	
Pyocyanine	−0·033	Friedheim and Michaelis [1931]
Indigotetrasulphonate	−0·046	Clark, cited from Michaelis [1933]
Indigodisulphonate	−0·125	
Lawson	−0·139	
Echinochrome	−0·220	Cannan [1927]
β-Anthraquinonsulphonate	−0·260	

D. *Juglon and lawson as natural oxidation-reduction indicators.*

In the husks of walnuts, juglon is generally found in the reduced form, lawson on the other hand exists in henna leaves in the oxidised state. If we consider the two pigments as natural intracellular oxidation-reduction indicators, we can infer that the oxidation-reduction potential of the plant-cells under consideration is more negative than the standard potential of juglon and more positive than the standard potential of lawson.

At $p_H = 7{\cdot}0$ this statement may be expressed as:

$$\underset{\text{juglon}}{+0{\cdot}033} > \underset{\text{cell}}{E_h} > \underset{\text{lawson}}{-0{\cdot}139}$$

or in terms of r_H

$$15{\cdot}1 > r_H \text{ cell} > 9{\cdot}2^1.$$

[1] Haas and Hill [1925] have described a plant pigment, hermidin, which has been shown by Cannan [1926] to behave like a reversible oxidation-reduction system. Calculated from the data given by Cannan, the standard potential would assume at p_H 7 a value of E_0 −0·033 and r_H 12·7. These figures fall well within the limits given above. It may be noted that the standard potential of hermidin coincides exactly with that of pyocyanine, the blue pigment of *Bacillus pyocyaneus*.

These limits, although far apart, are important because they hold good under physiological conditions, *e.g.* in air. They refer to the actual equilibrium between processes of oxidation and reduction in the living cell. This equilibrium is the result not only of thermodynamic but also of kinetic conditions, *i.e.* of diffusion and reaction velocities.

We owe our present knowledge of intracellular oxidation-reduction potentials up to date to the works of Needham and Needham [1925], Cohen *et al.* [1928], Wurmser and Rapkin [1931], *etc.* who have used the method of direct introduction of indicators into the living cell. It is important to supplement this method by means of direct observation of natural intracellular oxidation-reduction indicators, as the latter method eliminates possible sources of error such as toxicity of the indicator, trauma of injection *etc.*[1]

E. *The influence of juglon and lawson on the respiration of red corpuscles (rabbits).*

The work of Barron and Harrop [1928], as well as that of Michaelis and Salomon [1929–30], goes to prove that reversible oxidation-reduction systems are capable of increasing the respiration of red corpuscles, if non-toxic, able to permeate and possessing a normal potential more positive than that of indigo-disulphonate. Juglon and lawson, which fulfil these conditions, both behave in accordance with the above-mentioned rule: under their influence the respiration of the erythrocytes of rabbits is several times increased (see Fig. 2).

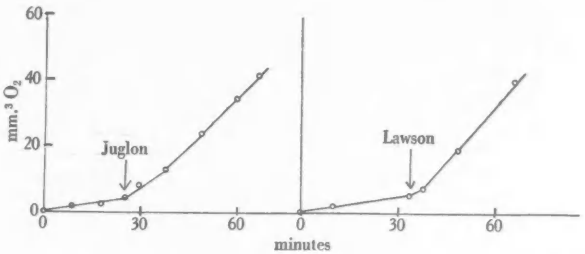

Fig. 2. The action of juglon and lawson on the respiration of red blood cells (rabbit).

Phosphate-Ringer p_H 7·2.
Phosphate concentration $M/75$.
0·2 % glucose.
Absorption of CO_2 by 5 % NaOH.
37° air.

At ↓ the dye is added from the side-bulb. Final dye concentration $M/1000$.

In our experiments juglon increases the respiration 5 times, and lawson 5·8 times. The effect of both these isomeric bodies is of the same numerical order, which however is difficult to reproduce exactly. It is independent of the nature of the medium (0·9 % NaCl, carbonate- or phosphate-Ringer) provided glucose is present.

[1] The conception of "intracellular" is as undefined as an address "London". In the various regions of the cell can be found different oxidation-reduction potentials (*e.g.* granula of oxidase and the "Sauerstofforte" of *Unna*). As fresh material is not available at present a more precise localisation of juglon and lawson will be attempted later on.

F. *The mechanism of increase of respiration in red corpuscles by reversible oxidation-reduction systems.*

Warburg *et al.* [1930] and Wendel [1929–30] have discovered the following mechanism for the increase of respiration of red corpuscles in the presence of methylene blue:

1. Methylene blue + haemoglobin + H_2O = methaemoglobin + leuco-methylene blue.

2. Methaemoglobin + carbohydrate = haemoglobin + oxidation products of carbohydrate.

3. Leuco-methylene blue + 1/2 O_2 = methylene blue + H_2O.

Michaelis and Salomon [1929–30] have further confirmed this theory for a number of reversible dyes and have brought it into correlation with the normal potentials of these dyes. The potential interval between indigodisulphonate and indigotetrasulphonate was defined as the critical level, on the positive side of which the increase in respiration runs approximately parallel with the formation of methaemoglobin. On the negative side, dyes are known to exist which can form methaemoglobin (indigodisulphonate, β-anthraquinonesulphonate, safranine) without exercising any influence on the respiration. This latter circumstance strengthens the view according to which the formation of methaemoglobin need not necessarily represent the crucial process in the increase of respiration by quinoid dyes.

Let us consider the conditions prevailing in the case of our hydroxynaphthoquinones: lawson and juglon both increase the respiration of the red corpuscles to considerable and approximately equal extents. But whereas the positive juglon, the normal potential of which is almost identical with that of methylene blue, rapidly oxidises haemoglobin to methaemoglobin, lawson, which is only a few mv. more negative than indigodisulphonate (at p_H 7) forms no methaemoglobin under conditions where an increase of respiration takes place. This has been ascertained from spectroscopic observation as well as from the determination of the oxygen capacity.

Table VII. *Oxygen capacity of one ml. of a suspension of red blood corpuscles (rabbits)**.

Temperature		mm.^{3} O_2
I. 25°	Control	61·5
	40 mins. after addition of M/1000 lawson (duplicate experiment)	(*a*) 61·7
	Spectroscopically: no methaemoglobin	(*b*) 62·0
II. 37°	Control	91·9
	40 mins. after addition of M/1000 lawson Spectroscopically: no methaemoglobin	91·6
	40 mins. after addition of M/1000 juglon Typical methaemoglobin bands at 635, shifted towards 610 under the influence of NaF	25·5 =28 %

* Method of Warburg *et al.* [1931].

Certain quinoid dyestuffs produce methaemoglobin without provoking any increase of respiration, whereas others, such as lawson, increase respiration without producing methaemoglobin. Thus the catalytic action of quinoid dyes on the respiration of red corpuscles is not indissolubly linked up with the action of the haemoglobin-iron.

The limits of potential of the juglon-hydrojuglon system overlap widely with those of the haemoglobin-methaemoglobin system. It is therefore possible for juglon to oxidise haemoglobin. At p_H 7·0 juglon, oxidised to approx. 99 % ($E_h = +0{\cdot}093$), forms a thermodynamical equilibrium with haemoglobin oxidised to about 50 % ($E_h = +0{\cdot}092 \pm 0{\cdot}022$). On the other hand, lawson, oxidised to the extent of 99 % ($E_h = -0{\cdot}079$) does not come within the range of the potential of a haemoglobin-methaemoglobin system reduced to the extent of 99 % ($E_h = -0{\cdot}028 \pm 0{\cdot}022$). The formation of methaemoglobin in the presence of lawson appears therefore rather improbable.

With regard to the formation of methaemoglobin by means of quinoid dyes, one should remember that the thermodynamical possibility, as expressed in terms of the standard potentials, is no guarantee that the reaction actually takes place.

β-Anthraquinonesulphonate oxidises haemoglobin: the more positive lawson ought to have at least a similar effect. But "kinetic factors outweigh thermodynamic factors in all reactions concerning haemoglobin" (Michaelis). Even after ten hours of exposure of red corpuscles to the action of lawson, no formation of methaemoglobin could be observed. Neither would a belated appearance of methaemoglobin be of any significance with regard to the mechanism of increase of respiration, as our experiments have shown that the latter itself appears immediately when lawson has been brought into contact with red corpuscles[1].

[1] I have described a number of natural reversible oxidation-reduction systems as "accessory respiratory enzymes." Stern [1933] questions the advisability of applying the term "enzyme" to the cases where methaemoglobin appears during the reaction. Whether or not these systems should be called enzymes depends entirely on the accepted definition.

Kuhn's definition in the work on ferments by Oppenheimer that "ferments are catalysts manufactured by the cell," holds good for the chemical bodies with which we are concerned, including juglon and lawson. If one accepts on the other hand Haldane's restriction of the definition to the effect that "ferments are colloidal catalysts," then these crystalline substances cannot be regarded as ferments.

By no means can we consider the nature of intermediate products, *e.g.* the possible formation of methaemoglobin, as an argument either for or against the enzymic character of the reaction.

As in this paper the formation of methaemoglobin has been shown to be irrelevant for the enhancing effect of reversible oxidation-reduction systems on respiration of red blood cells, Stern's discussion appears all the more beside the point.

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XXVII. A NOTE ON THE PRESENCE OF VITAMIN C IN THE CHICK EMBRYO.

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(*Received December 23rd, 1933.*)

It has long been known that fowls and other birds resemble rats [Parsons, 1920] and dogs [Harris, 1931] in having little apparent need for vitamin C in their diet. Plimmer *et al.* [1923] concluded that the growth of the chick in the egg, which is known to be free from vitamin C, is one of the best indications that vitamin C is not needed by the growing bird. This statement has been criticised on the ground that the avian embryos may synthesise the vitamin during their development, and indeed Carrick and Hauge [1925] and Hart *et al.* [1925] demonstrated the presence of vitamin C in the livers of chicks which had been fed for a long time on vitamin C-free diets. Hauge and Carrick [1926] further showed that though both the yolks and the whites of eggs laid by hens which had been on scorbutic ration contained no demonstrable amount of vitamin C, yet the livers of five-weeks-old chicks hatched from these eggs and fed on scorbutic rations contained an abundance of this vitamin. This makes it evident that during incubation vitamin C is being produced in the growing embryo.

The earlier studies, which involved the employment of large amounts of embryonic tissue for biological assay, prevented the actual detection of vitamin C in the growing embryo. The much simpler micro-chemical method of Birch *et al.* [1933] makes the demonstration of the vitamin an easy and rapid affair.

Experimental.

Brown Leghorn hens' eggs were used. The eggs after the proper time of incubation were opened, the embryos removed and dried on filter-paper. They were rapidly weighed and ground up with a little sand and sufficient 20 % solution of trichloroacetic acid to give a final concentration of the latter of about

Table I.

Day of incubation	Weight of the substance taken g.		Total amount of ascorbic acid mg.	Amount of ascorbic acid in mg./g. wet tissue	Amount of ascorbic acid in mg./g. dry tissue
0	(*a*) Egg-white	27	0	0	0
	(*b*) Egg-yolk	20	0	0	0
4	(*a*) 5 whole embryos	0·6	0·0745	0·124	2·98
	(*b*) Egg-white	11·6	0	0	0
	(*c*) Egg-yolk	20	0	0	0
6	2 whole embryos	0·89	0·143	0·160	3·04
8	1 ,,	1·17	0·213	0·182	2·91
10	1 ,,	2·68	0·630	0·235	3·38
12	1 ,,	5·05	1·260	0·249	2·81
15	Embryo livers	0·50	0·132	0·264	—
17	,,	0·37	0·105	0·283	—
19	,,	0·65	0·178	0·274	—
21	,,	0·82	0·226	0·275	—
24 hours old chick-liver		1·05	0·273	0·260	—

5 %. The extract was then made up to a suitable volume and the filtrate titrated against a measured volume of a standard solution of 2 : 6-dichlorophenolindophenol. Up to the 15th day the whole embryos were used; on the 15th day and after, the livers only were examined.

The dry weight of the embryo, used to calculate the figures in the last column of the table, was obtained from Murray's [1926] figures for chick embryo. As the wet weights were found to be nearly identical with his values of wet weight of the embryo, it may be taken that the figures in the last column are reasonably accurate.

Discussion.

It is evident from Table I that while there is no reducing substance in the fresh hens' eggs, quite large amounts of such substances appear in the embryo on incubation. It is also known from previous work [Birch *et al.* 1933; Harris, 1933] that only a few substances other than ascorbic acid reduce the dye under the conditions of the experiment. As these other substances are not likely to be present in the fresh embryo tissue, the amount of reduction can be taken to be a fair indication of the amount of vitamin C present. Further, Hart *et al.* [1925] found that 3 g. of liver from a chicken kept on scorbutic ration for 83 days, constitute a curative dose for scorbutic guinea-pigs; while 1 g. of such liver is definitely not sufficient. If we now take 0·9 mg. of ascorbic acid as the minimum curative dose [Harris and Ray, 1933, 1, 2] and take from Table I the average amount present in liver to be 0·26 mg., the minimum curative dose for chicken liver works out to be 3·4 g. This value is in close agreement with the figures given by Hart *et al.* [1925].

The appearance of vitamin C with the commencement of growth is also seen in the germination of seeds. Moreover, it may be seen from Table I that the amount of vitamin C present per g. of the dry embryo tissue is fairly constant. These facts naturally suggest that there may be some connection between the two phenomena—the appearance of vitamin C and the beginning of growth. In order to determine whether excess of vitamin C has any influence on the rate of growth, some preliminary experiments were performed by injecting about 2 mg. of ascorbic acid (ten times the amount present in the developing embryo) in sterile aqueous solution into the air-space of the eggs before incubation. The result was however totally negative. From both the untreated and treated eggs, the chickens came out simultaneously after the usual incubation period of 21 days, nor was there any great difference between the weights of the two classes. It does not seem likely therefore that moderate excesses of vitamin C have any further direct action on the growth.

Summary.

1. The embryo of the chick has been examined for vitamin C. There is no vitamin present in the fresh egg, though quite a large amount is present even after only 4 days' incubation. The amount of vitamin C present in embryo chick liver agrees very well with the value given by Hart *et al.* [1925] for livers of chickens kept for a long time on a scorbutic ration.

2. Aqueous solutions of vitamin C when injected into the hen's egg do not seem to produce any change in the growth-rate of the embryo.

My thanks are due to Sir F. G. Hopkins for his kind interest in this work. I am indebted also to Dr L. J. Harris and Dr J. Needham for their constant advice and encouragement.

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XXVIII. THE ENZYMIC FORMATION OF HYDROGEN SULPHIDE BY CERTAIN HETEROTROPHIC BACTERIA. II.

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(*Received January 1st, 1934.*)

In a previous communication [Tarr, 1933] the formation of hydrogen sulphide from organic sulphur compounds by certain heterotrophic bacteria was attributed to the activity of a specific enzyme present in their cells. Evidence was also obtained in support of the fact that different mechanisms are responsible for the formation of hydrogen sulphide from organic sulphur compounds and from thiosulphate by such bacteria. This evidence has been substantiated by the results obtained in an experiment described in the present paper. It is possible that the formation of hydrogen sulphide from inorganic sulphur compounds is also due to different mechanisms. Thus the sulphate bacteria produce hydrogen sulphide from sulphate, sulphite and thiosulphate [Stephenson and Stickland, 1931], and from hyposulphite and sulphur as well [Baars, 1930], while *P. vulgaris* cells only reduce thiosulphate and sulphur to hydrogen sulphide [Tarr, 1933]. The experimental work described in this paper has been almost entirely confined to the formation of hydrogen sulphide from organic compounds.

Experimental.

The technique employed was essentially the same as that previously described [Tarr, 1933]. Unless otherwise stated all experiments were made anaerobically in evacuated 75 ml. Thunberg tubes, each tube containing 5 ml. of bacterial suspension, 2 ml. of $M/5$ phosphate buffer, p_H 7·8, the required amount of substrate solution and a final fluid volume of 20 ml. Strictly aseptic precautions were only observed in experiments the duration of which was over 2 hours.

The homocystine used was kindly given to me by Prof. V. du Vigneaud, the dithiodihydracrylic acid (disulphide from β-thiolpropionic acid) by Dr A. Schöberl, and the *dl*-cysteine hydrochloride by Dr N. W. Pirie. Solutions of these compounds were prepared in accordance with the general procedure previously described [Tarr, 1933].

The formation of hydrogen sulphide from cysteine and from thiosulphate by P. vulgaris *cells.*

It was previously shown [Tarr, 1933] that hydrogen sulphide is formed from cysteine by cells of both *P. vulgaris* and *S. marcescens*, but from thiosulphate only by the former organism, thus indicating that two different systems capable

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of forming hydrogen sulphide from these substrates exist. The following experiment shows that *P. vulgaris* cells possess both systems.

Ten Thunberg tubes containing buffer and *P. vulgaris* cells as usual were prepared. One pair of tubes received 5 mg. of $Na_2S_2O_3$ per tube, a second pair 6 mg. of $Na_2S_2O_3$, a third pair 1 mg. of cysteine, and a fourth pair 5 mg. of $Na_2S_2O_3$ *plus* 1 mg. of cysteine. A fifth pair was retained as control. The tubes were evacuated as usual and placed in a water-bath at 37° for 3½ hours, and the amount of hydrogen sulphide formed was then determined. The results of this experiment, which are given in Table I, show that the addition of cysteine to

Table I. *The effect of the addition of cysteine on the production of H_2S by* P. vulgaris *cells saturated with respect to $Na_2S_2O_3$.*

Experiment	H_2S in γ after 3½ hours at 37°	
	Duplicates	Average
5 mg. $Na_2S_2O_3$	67 69	68
6 mg. $Na_2S_2O_3$	70 74	72
1 mg. cysteine	67 65	66
1 mg. cysteine + 5 mg. $Na_2S_2O_3$	126 126	126

23 mg. dry weight of bacteria per exp. Controls formed no H_2S.

P. vulgaris cells which are saturated with respect to thiosulphate results in an increase in hydrogen sulphide formation almost equivalent (91 %) to the sum of the hydrogen sulphide formed from cysteine and thiosulphate alone. This may be considered practically conclusive proof that H_2S formation from thiosulphate and from cysteine by *P. vulgaris* is due to two entirely different mechanisms.

The action of P. vulgaris *and* S. marcescens *cells on* l-*cysteine,* dl-*cysteine, homocystine and the disulphide from β-thiolpropionic acid.*

The technique employed was identical with that previously used in studying H_2S formation from different compounds [Tarr, 1933], and the results of the experiment are given in Table II. The action on *l*-cysteine was studied in order that the results obtained might be strictly comparable with those recorded in the previous paper. Although the disulphide from β-thiolpropionic acid is reduced to the —SH form by both organisms, as is evidenced by a strong nitroprusside reaction, neither of them forms H_2S from it. On the other hand homocystine is reduced by both bacteria, with simultaneous formation of H_2S in small amounts. *S. marcescens* forms H_2S from *l*-cysteine and from *dl*-cysteine equally well, but *P. vulgaris* exhibits greater activity in this respect toward the former compound. The facts that most of the sulphur of cysteine can be recovered as H_2S, while none of the sulphur of the corresponding acid which lacks an amino-group yields H_2S, make it appear highly probable that the presence of an amino-group, and consequently deamination, is necessary to the successful course of the reaction. Thus homocystine which contains an amino-group yields H_2S under the experimental conditions in spite of the fact that the —SH group is on the γ-carbon atom.

Table II. *The anaerobic formation of H_2S from* l-*cysteine,* dl-*cysteine, homocystine and the disulphide from β-thiolpropionic acid by washed cells of* P. vulgaris *and* S. marcescens.

Compound	Weight used mg.	Theoretical amount of H_2S in mg.	H_2S recovered: *P. vulgaris* mg.	*P. vulgaris* %*	*S. marcescens* mg.	*S. marcescens* %*
l-Cysteine	0·5	0·141	0·120 0·115	79	0·120 0·112	79
dl-Cysteine	0·5	0·141	0·073 0·075	49	0·115 0·120	80
Homocystine	0·5	0·126	0·033 0·038	23	0·018 0·020	12
Disulphide from β-thiolpropionic acid	0·5	0·162	0·006 0·006	0	0·004 0·004	0
Controls	0·0	0·0	0·006 0·006	0	0·004 0·004	0

* Calculated on the theoretical value after subtracting the control.

P. vulgaris 28 mg. dry weight of bacteria per exp.
S. marcescens 54 ,, ,,

The extraction from P. vulgaris *cells of a cell-free preparation of the enzyme forming H_2S from cysteine.*

The procedure to be described was conducted under as nearly aseptic conditions as possible in order to avoid the presence of contaminating bacteria in the final preparations. The suspensions of *P. vulgaris* cells were prepared as usual, the cells being suspended in distilled water instead of Ringer solution, so that a suspension containing about 50 mg. dry weight of bacteria in 10 ml. was obtained. Suspensions prepared in this manner were run slowly, and with rapid stirring, into 10 volumes of acetone. The resulting flocculent precipitate was permitted to settle and the supernatant liquid carefully syphoned off, and a second 10 volumes of acetone were added. The precipitate was then collected on a small Büchner funnel, pressed between sterile filter-papers and dried for 2–3 hours at 28°. The resulting dry brittle mass was used within 2 days of preparation.

The acetone-dried bacteria were ground to a fine powder in a small mortar and mixed with $M/20$ phosphate buffer (p_H 7·8), 10 ml. being used for each 100 mg. of bacteria. The resulting suspension (A) was incubated for 2 hours at 37° and then centrifuged for 1 hour at high speed. The faintly opalescent, pale yellow supernatant liquid (B) was decanted, and the precipitate (C) was resuspended in distilled water and made up to a volume equivalent to that of the original suspension. Some of liquid B was filtered through a layer of kieselguhr, and some through a Seitz asbestos filter, and the resulting almost water-clear solutions labelled (D) and (E) respectively. A portion of the kieselguhr filtrate was heated to 80° for 2 minutes (F). The ability of these various fractions to form H_2S from 10 mg. of cysteine was determined, corresponding controls without cysteine being run in each case. The results, which are given in Table III, show that suspensions of acetone-treated bacteria form small amounts of H_2S, though calculation has shown that such preparations possess only 2–3 % of the original activity of the freshly prepared cells. On centrifuging suspensions of acetone-bacteria about one-half of the active principle remains in the supernatant liquid, while the remaining half is to be

found in the precipitate. Filtering the supernatant liquid through kieselguhr yields a clear, cell-free fluid, which retains the activity of the original liquid in full. On the other hand all the activity is lost by filtering the solution through a Seitz filter. Heating to 80° for 2 minutes renders kieselguhr filtrates inactive.

Table III. *The formation of H_2S by extracts of acetone-dried* P. vulgaris *cells.*

Preparation (10 ml. per exp.)	H_2S in γ produced from 10 mg. of cysteine in 4 hours at 37°
A	45
B	20
C	23
D	19
E	0
F	0

Controls gave no H_2S. Bacterial examinations showed that all solutions prepared under such conditions are sterile or nearly so.

All attempts to obtain active cell-free enzyme preparations by autolysing *P. vulgaris* cells for a week at 37° in phosphate buffer of p_H 7·8 or by drying cells *in vacuo* and extracting them with phosphate buffer have proved unsuccessful.

The properties of the enzyme forming H_2S from cysteine as determined by the use of intact P. vulgaris *cells.*

The effect of p_H on the velocity of H_2S formation from cysteine was determined. Phthalate-NaOH and KH_2PO_4-NaOH buffers (Clark and Lubs), and a glycine-NaCl-NaOH buffer (Sørensen) were employed. (Unpublished experiments have shown that *P. vulgaris* cells do not decompose glycine under anaerobic conditions.) The amount of H_2S formed from 1 mg. of cysteine in 4 hours at 37° was estimated at various p_H values, 2 ml. of the required buffer solution being employed in each experiment. The H_2S formed in the absence of cysteine under identical conditions was subtracted in each case. The results of this experiment are shown in Fig. 1. A study of this figure indicates that the

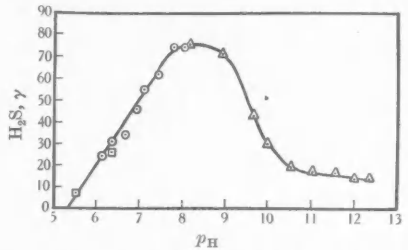

Fig. 1. The p_H-activity curve of the enzyme forming H_2S from cysteine.
⊡ Phthalate-NaOH buffer. 34 mg. dry weight of bacteria per experiment.
⊙ KH_2PO_4-NaOH buffer.
△ Glycine-NaCl-NaOH buffer.

reaction proceeds with maximum velocity between p_H 7·8 and 9·0, there being a rapid decline in activity at higher and lower p_H values respectively. The enzyme is inactive at p_H 5·5. Curiously enough the enzyme is still active at p_H 12, although no H_2S is obtained from cysteine alone at this p_H under the

experimental conditions. Additional experiments have shown that if washed *P. vulgaris* cells are held at 37° for 5 minutes in *M*/20 glycine-NaCl-NaOH buffer of p_H 12 and the p_H then adjusted to approximately 7·8, about 37 % of the original activity of the cells remains. Thus in one experiment untreated cells formed 80γ of H_2S from 10 mg. of cysteine in 1 hour at 37°, and cells treated as above described formed 30γ of H_2S under similar conditions.

In Fig. 2 the effect of temperature on the amount of H_2S formed from 1 mg. of cysteine in 4 hours is shown. It is evident from this figure that the reaction has a relatively sharp optimum temperature of about 40°, and that an extremely rapid decline in activity is exhibited at higher or lower temperatures. The fact that the enzyme is inactive at 50° indicates that it is fairly sensitive to heat.

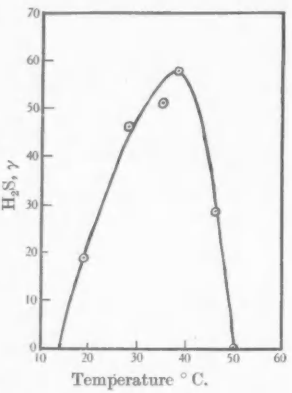

Fig. 2. The effect of temperature on the velocity of H_2S formation from cysteine by *P. vulgaris* cells.

26 mg. dry weight of bacteria per experiment.

The influence of aerobic and anaerobic conditions on the velocity of H_2S formation from 10 mg. of cysteine in $\frac{1}{2}$ hour at 37° was determined. In one experiment the Thunberg tubes were evacuated in the usual manner, in a second a rapid stream of nitrogen washed thoroughly through alkaline pyrogallol solution was bubbled through the solutions during the course of the reaction, and in a third a stream of air was used in place of nitrogen. In the last two of these experiments the H_2S formed was absorbed in zinc acetate solution during the course of the reaction. In all experiments after half an hour the experimental solution was acidified with H_2SO_4 in the usual manner, and the H_2S present aerated into the zinc acetate solution. The results of these experiments, recorded in Table IV, indicate that H_2S is formed much more rapidly from cysteine under aerobic than under anaerobic conditions, this result being in agreement with the data obtained by Almy and James [1926] for *P. vulgaris* grown in a cystine-containing medium. In addition it is evident from Table IV that the velocity of H_2S formation is greater under the anaerobic

Table IV. *The effect of aerobic and anaerobic conditions on the formation of H_2S from cysteine by* P. vulgaris.

	H_2S in γ after $\frac{1}{2}$ hour at 37°	
Conditions of experiment	Duplicates	Average
Aeration with air	141 141	141
Nitrogen passed through the solutions	80 80	80
Evacuated tubes	60 58	59

Controls without cysteine gave no H_2S. 22 mg. of dry weight of bacteria per exp.

conditions created by passing a stream of nitrogen through the experimental liquid than under similar conditions created by a partial vacuum. It is possible that this last-named effect is due to the fact that in experiments in which

nitrogen is employed the reaction product (H_2S) is more rapidly removed from the seat of the reaction than in experiments *in vacuo*.

The influence of cyanide on the velocity of H_2S formation was studied, 10 mg. of cysteine being employed in each experiment. All experiments were carried out at 37°, H_2S being estimated after ½ and 1 hour. 5 ml. of *M*/5 phosphate buffer of p_H 7·8 were used instead of the usual 2 ml. The effect of cyanide was investigated by placing a small tube containing 1 ml. of KCN solution of appropriate concentration in the fluid in the Thunberg tube employed and mixing the liquids subsequent to evacuation. It is evident from the results given in Table V that KCN in the concentrations employed causes only a partial inhibition of the reaction. The inhibition due to a given cyanide concentration decreases with time, and the reaction is, therefore, reversible.

Table V. *The effect of KCN on the velocity of H_2S formation from cysteine by* P. vulgaris.

	H_2S formed in γ from 10 mg. cysteine			
Experiment	½ hour	% inhibition	1 hour	% inhibition
Controls (no KCN)	84	—	120	—
	84		126	
M/1000 KCN	60	29	100	20
	60		95	
M/500 KCN	36	56	76	37
	38		78	

24 mg. dry weight of bacteria per exp. Controls without cysteine gave no H_2S.

A study was also made of the effect of toluene treatment, 5 % ethylurethane and phenylurethane (saturated solution) on the velocity of H_2S formation from cysteine. The toluene-treated bacteria were prepared according to the method of Quastel and Wooldridge [1928], and all attempts to obtain growth of bacteria from such suspensions on caseinogen digest agar were unsuccessful. The results of these experiments (Table VI) show that phenylurethane causes no inhibition

Table VI. *The effect of toluene treatment, phenylurethane and ethylurethane on the velocity of H_2S formation from cysteine by* P. vulgaris.

Experiment	H_2S in γ from 10 mg. cysteine in 1 hour at 37°	% inhibition
Controls	97	0
	97	
Toluene-treated cells	54	45
	54	
5 % ethylurethane	36	62
	38	
Saturated phenylurethane	95	0
	100	

34 mg. dry weight of bacteria per exp. Control exps. without cysteine gave no H_2S.

of the reaction, while 5 % ethylurethane inhibits the reaction by about 60 %. It is interesting to note that toluene-treated *P. vulgaris* cells, which are "non-viable" as far as ability to reproduce on culture media is concerned, still retain over 50 % of their ability to form H_2S.

SUMMARY.

1. Hydrogen sulphide formation from cysteine and from thiosulphate by washed *P. vulgaris* cells is due to two different mechanisms. The formation of H_2S from organic sulphur compounds by this bacterium is due to the activity of a specific enzyme present in its cells.

2. Sterile, cell-free extracts containing small amounts of this enzyme can be obtained by extracting acetone-dried *P. vulgaris* cells with phosphate buffer, centrifuging and filtering the resulting supernatant liquid through kieselguhr. Such extracts exhibit only 2–3 % of the original activity of the cells from which they are prepared.

3. This enzyme as it exists in intact *P. vulgaris* and *S. marcescens* cells apparently requires the presence of an amino-group before it can become active. Thus H_2S is formed from α-amino-β-thiolpropionic acid (cysteine) but not from β-thiolpropionic acid, while α-amino-γ-thiolbutyric acid (homocysteine) gives rise to small amounts of H_2S.

4. The enzyme as it exists in intact *P. vulgaris* cells is inactive at p_H 5·5, is most active at p_H 7·8–9·0, and retains nearly 20 % of its optimum activity at p_H 12. It has an optimum temperature of 40° and is inactive at 50°. More H_2S is formed in a given time by the enzyme under aerobic than under anaerobic conditions. Non-viable, toluene-treated bacteria retain over 50 % of their activity with respect to H_2S formation. Phenylurethane does not inhibit H_2S formation from cysteine, while 5 % ethylurethane causes some 60 % inhibition of this reaction. Cyanide only partially inhibits the reaction, and this inhibition is reversible.

I am greatly indebted to Prof. V. du Vigneaud of George Washington University for his kindness in giving me the homocystine used in this work, to Dr A. Schöberl of the Chemisches Inst. der Univ. Würzburg for a supply of dithiodihydracrylic acid, and to Dr Pirie of this Department for the *dl*-cysteine hydrochloride employed. For his constant interest and encouragement I wish to thank Sir F. G. Hopkins.

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XXIX. THE DETERMINATION OF THE MEMBRANE POTENTIALS OF PROTEIN SOLUTIONS AND THE VALENCE OF PROTEIN IONS.

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(*Received January 1st, 1934.*)

1. *Introduction.*

The theoretical treatment of membrane potentials in very dilute solutions is mainly due to Donnan [1911], who considered a system in which a solution containing the sodium salt of a colloid such as Congo red is separated from a solution of sodium chloride by a membrane permeable by water and crystalloids but impermeable by the colloidal particles. After equilibrium has been reached, the concentration of sodium ions is greater in the solution inside the membrane, containing the colloid, and there is a potential difference between the colloidal solution and the salt solution or dialysate, determined by Nernst's formula

$$\frac{E}{1000}=\frac{-RT}{F}.ln\frac{[\mathrm{Na}]'}{[\mathrm{Na}]''}=\frac{-2{\cdot}303RT}{F}\log\frac{[\mathrm{Na}]'}{[\mathrm{Na}]''} \qquad \text{.....(1).}$$

E = the potential difference or membrane potential in millivolts. $F = 96{,}494$ coulombs. $R = 8{\cdot}316$ volt coulombs. T = the absolute temperature. $[\mathrm{Na}]'$ = the concentration of sodium ions in the colloidal solution. $[\mathrm{Na}]''$ = the concentration of sodium ions in the dialysate.

The sign of the potential is the same as the sign of the colloidal particle, negative in the case of Congo red and positive in the solutions of protein chlorides studied by Loeb [1922], who devised a method for the experimental investigation of membrane potentials.

An extension of the theory to more concentrated solutions was made by Donnan and Allmand [1914], who derived a formula in which the concentrations in equation (1) are replaced by effective concentrations or activities.

Brønsted [1929], Taylor, P. B. [1927] and Guggenheim [1929] have emphasised the difficulty of measuring the potentials between two different media and the activities of individual ions, but in colloidal solutions the molar fraction of water is so nearly equal on both sides of the membrane that the conditions for the application of the formula of Donnan and Allmand are more favourable, even if absolute accuracy is unattainable on account of liquid junction potentials.

In view of the number and variety of the practical applications of measurements of membrane potentials in colloidal systems, even approximate results may be of great value, and in certain cases it is possible to compare the results with data obtained by methods that are independent of liquid junctions, including measurements of osmotic pressure and determinations of the concentrations of ions on both sides of the membrane by chemical methods.

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Determinations of p_H values, membrane potentials, combined ions and other properties of proteins deduced from measurements in which approximate elimination of liquid junction potentials has been achieved by the use of saturated solutions of potassium chloride must be regarded as conventional rather than absolute values.

The chief application of membrane potentials described in this paper is the estimation of the electric charge or valence of the protein ions from the limiting value of the ratio of the membrane potential to the protein concentration. A preliminary note on the method has been included in a previous paper [Adair and Robinson, 1930, 2].

2. *Liquid junction potentials.*

Sørensen and Linderstrøm-Lang [1924] have found that in most of the solutions that are of biological interest the elimination of the liquid junction potentials by the use of saturated potassium chloride gives the same result as the Bjerrum extrapolation. Bjerrum and Unmack [1929] have made an extensive series of calculations of the liquid junction potentials with 3·5 N potassium chloride by the application of Henderson's formula. If the concentration of ions like sodium, with mobilities different from the potassium and chloride ions, be less than 0·2 M, the potentials rarely exceed 1 millivolt, and even if hydrogen and hydroxyl ions be present in concentrations up to 0·03 M the potentials are usually within 1·2 millivolts. The choice of solvents within this range is of importance from the experimental point of view in reducing variations in the potentials due to differences in the methods of forming the liquid junctions, so that reproducible results can be obtained by methods specially adapted for measurements on small volumes of solutions. In order to obtain reproducible results with markedly acid or alkaline solutions, it may be necessary to use flowing junctions [Lamb and Larson, 1920] or static junctions in wide tubes [Ferguson *et al.*, 1932].

In previous investigations on membrane potentials, liquid junctions in capillary tips have been used by Loeb [1922] and by Taylor [1924], in order to minimise the contamination of the solution with potassium chloride. Most investigators have used wider tubes, which give more reproducible liquid junctions. Kondo [1924] formed the junction at the end of a wide tube, Bjerrum made liquid junctions in glass taps, Northrop and Kunitz [1926] formed stirred junctions in wide tubes.

Different types of liquid junctions have been discussed by Guggenheim [1930]. He criticises the practice of stirring junctions, on the ground that it forms two junctions of indefinite type between the first solution and the mixture and the second solution. He advises the use of sharp junctions formed inside tubes and not at their tips. Maclagan [1929] compared junctions formed with tubes of different diameters and concluded that junctions formed inside tubes are more reproducible.

Stadie and Hawes [1928] showed that the liquid junction formed in the Clark hydrogen electrode gives reproducible results in measurements of the p_H values of mixtures containing haemoglobin and sodium salts. This method has been employed in this work in the determinations of membrane potentials from p_H values.

3. *The experimental determination of membrane potentials.*

In the method for the measurement of membrane potentials devised by Loeb [1922], a solution of the protein contained in a 50 ml. collodion membrane, with a manometer attached, was placed in a vessel of "outer fluid" or dialysate,

and the system was left for 20 hours to reach equilibrium. The membrane potential was determined by dipping saturated calomel-KCl electrodes into the dialysate and the protein solution, which was forced up into a funnel by pressure on the flexible collodion membrane. In most of Loeb's experiments the results obtained were verified within two millivolts by their ageement with values calculated by the application of Donnan's formulae to measurements of the activities of hydrogen ions on both sides of the membrane.

In the modifications of the methods devised by Bjerrum [1924] for chromium chloride, by Kondo [1924] for caseinogen chloride and by Henriques [1928; 1929] for laked blood corpuscles, the liquids were enclosed, and long glass tubes connected the inner and outer fluids with calomel electrodes. These designs facilitate the measurement of potentials at short intervals of time; moreover, the pressures may be adjusted at the values corresponding to osmotic equilibrium. According to Rinde [1926], measurements of potentials must be made at the equilibrium pressure, whereas Northrop and Kunitz [1926] consider that it is unnecessary to maintain the equilibrium pressure during the measurement. As will be stated below, our experience agrees with that of Northrop and Kunitz.

The method for the determination of membrane potentials to be described in this paper was developed for work on purified protein solutions, in order to obtain consistent results under conditions where the potentials may be small and the volumes of solution available for each determination may be restricted to 5 or even 1·5 ml. The types of apparatus used in this work are not adapted for making continuous records of the potentials, but they have the advantage that dead spaces, which may contain protein not in equilibrium with its dialysate, are practically eliminated, and the distances for diffusion are comparatively short (Fig. 1).

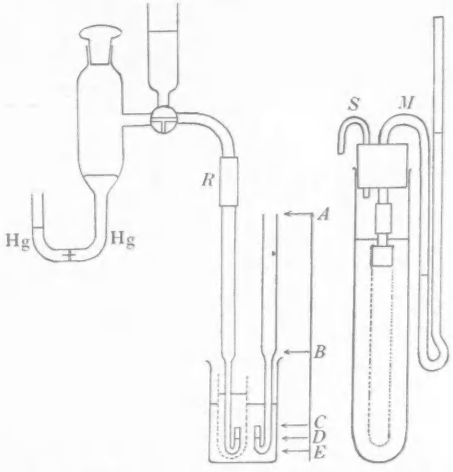

Fig. 1. Apparatus for measurement of membrane potential of small volumes of protein solutions with saturated calomel-KCl electrodes.

Collodion membrane containing protein shown by dotted lines. *R* is rubber connecting tube. Electrode tip, *A* to *B* 90 mm. tube of 5 mm. internal diameter; *B* to *E*, 55 mm. tube of 2 mm. internal diameter; *E* to *C*, 15 mm. Liquid junction at *D*, 7 mm. below orifice at *C*. *M* is mercury manometer bore 1·5 mm. attached to osmometer used for equilibration. *S* is for attachment of soda lime tube.

The establishment of equilibrium between the protein solution and the dialysate is the first consideration in studies of membrane potential. At ordinary temperatures, rapid equilibration may be necessary to prevent errors due to changes in the protein and to bacterial action. In the present research, the work throughout was carried out at 0°, and since bacterial action is inhibited and the stability of the proteins is greater at low temperatures, it has been possible to make more rigorous tests of equilibrium. Instead of leaving given volumes of protein solution and dialysate to reach equilibrium, as described by Loeb, the protein solutions were equilibrated by dialysis against relatively large volumes of solutions of well defined hydrogen ion concentration, in order to control the activities of the electrolytes in the protein solutions and to facilitate the thermodynamical treatment of the systems [Adair, 1929, 1, 2]. The standard buffer solutions remove traces of inorganic impurities which are not readily eliminated by a preliminary dialysis against distilled water; the rate of removal of traces of an acid or base combined with the protein is very greatly increased if the dialysate be a solution of electrolytes rather than pure water.

Method 1. In the majority of the experiments to be described in this paper, the process of equilibration has been followed by observation of the osmotic pressure.

The collodion membranes used were prepared from a solution containing 4 g. of dry pyroxylin (B.D.H.) dissolved in a mixture of 50 ml. of absolute alcohol and 50 ml. of anhydrous ether, or by diluting one volume of "Necol" with one volume of alcohol and one volume of ether. The membranes were made by pouring three coats of collodion over the outside of a glass mould [Adair, 1925; Cole, 1933]; they were therefore less permeable but more rigid and probably more uniform than those used by Loeb, which were made on the inside of a tube with one coat of collodion. The permeability may be altered by varying the time allowed for drying the collodion. The membranes were mounted on rubber tubes or stoppers, and the permeability was tested by measuring the rate of flow of water through the membrane at room temperature under a pressure of 450 mm. Hg. For a cylindrical membrane of 10 cm. length and 1·5 cm. diameter, the rate of flow should range from 0·05 to 0·2 ml. of water per minute. Membranes of higher permeability may allow an appreciable leakage of a protein such as haemoglobin, whereas those which are less permeable entail an unduly prolonged period of equilibration.

The collodion membranes were attached to mercury manometers of the type illustrated in Fig. 1 for concentrated solutions, and of the type described by Adair [1925] for more dilute solutions. The dialysates were changed at intervals of one or two days until further changes caused no further alterations in the manometer readings. If the volume of the dialysate be about ten times as great as the volume of the protein solution, a close approximation to equilibrium is reached after three or four changes, in the case of well buffered solutions.

If the buffer solutions used are more dilute than 0·3 M, it is advisable to dialyse the protein solutions for two or three days against smaller volumes of buffer, changed at short intervals, before the membranes are attached to manometers, and if there be a large difference between the initial hydrogen ion concentration of the protein solution and the equilibrium value, it may be advisable to adjust the p_H of the protein solution or to add a slight excess of acid or base to the dialysate in the early stages of dialysis.

The apparatus and procedure may require modification for work on very dilute solutions (0·001 M), or for solutions in which the p_H values are altered by a short exposure to air.

The determinations of membrane potential were made after equilibrium had been reached and maintained for a few days. Closed saturated calomel-KCl electrodes with upcurved tips were used, of the design shown in Fig. 1. The electrodes were kept throughout at 0° and were connected by leads to a potentiometer in the laboratory outside. A mirror galvanometer of 450 ohms resistance was used, giving a deflection of about 40 mm. per millivolt, with electrodes dipped into solutions of high conductivity. A multiple range potentiometer was used for some experiments, but as a general rule the measurements were made with a simpler instrument, graduated in 0·2 millivolt.

The difference between the two calomel cells was first determined with both electrode tips placed in a tube of saturated KCl; the electrode tips were then washed with distilled water and dried with filter-paper, and the blank determination was repeated. The two measurements should not differ by more than 0·01 millivolt, but variations of 0·03 millivolt have been observed if the calomel cells have been shaken during the manipulations. If the blank determinations agreed, the electrodes were again washed with distilled water, and a few drops of KCl were run out to displace water which might have entered the tips. A small quantity of KCl was then forced out by pressure on the rubber tube connecting the electrode tip and the calomel cell, so that the level of KCl was about 7 mm. below the orifice; the electrodes were then dried with filter-paper. After both electrodes had been prepared, an osmometer was dismantled and the protein solution filling the dead space, *i.e.* that which had not been in contact with the membrane, was rejected. The bulk of the protein was removed for analysis, a depth of solution of 2 to 3 cm. being left in the membrane. The rubber mount was then cut off, and the membrane was placed in a small beaker containing dialysate. In cases where a positive charge on the protein was anticipated, the more positive of the two calomel cells was selected, and the orifice of the electrode tip, from which KCl had been removed, was filled with the protein solution; the other electrode was filled with dialysate. Both electrodes were then washed with dialysate and dried with filter-paper. The more positive electrode was then immersed in the protein solution remaining in the membrane and the other electrode in the dialysate.

In order to avoid the formation of air bubbles in filling the electrode tips, the rubber connecting tube was pressed until KCl rose to the level of the orifice. The protein solution could then be drawn into the tip from a capillary pipette by releasing the pressure on the rubber tube.

The first reading of the potential could generally be obtained between three and four minutes after the formation of the liquid junctions. The reproducibility of the junctions formed by this method and the variation of the reading with time are discussed in the following section.

Method 2. In this method, the protein solutions were contained in collodion membranes mounted on celluloid tubes of 1·5 cm. length. During equilibration, the membranes were closed with rubber stoppers and were inverted at intervals. The attainment of equilibrium was determined by measuring the potential difference between protein solution and dialysate on successive days and renewing the dialysate each day until further changing caused no alteration in the potential. Stoppered pyrex beakers were used for equilibration.

The osmotic method is more convenient for series of more than twelve membranes, as the manometer readings can be made more rapidly than a single estimation of membrane potential. The second method is useful for the measurement of small potentials, as a number of determinations can be obtained from each membrane, and the effects of certain sources of error, including changes in

the E.M.F. of the calomel cells and variations in the liquid junction potentials, can be minimised by taking the average of a number of observations after equilibrium has been reached. Observations on the rate of equilibration of haemoglobin solutions with 0·02 *M* phosphate buffers, obtained by this method, are given in Table I.

Table I. *Rate of equilibration of 22 % solutions of sheep carbon monoxide haemoglobin with 0·01* M $NH_4H_2PO_4 + 0{\cdot}01$ M $(NH_4)_2HPO_4$ *at 0°, shown by measurements of membrane potential on successive days.*

Permeabilities in ml. water per minute at a pressure of 450 mm. of mercury for membranes 4·5 cm. long and 1·4 cm. diameter. Times in days. Potentials in millivolts.

Membrane permeability	1	4	6	8	12	21	55 days
0·086	−1·35	−0·77	−0·66	−0·62	−0·54	−0·55	—
0·066	−1·20	−0·72	−0·64	−0·66	−0·46	−0·55	—
0·102	−1·15	−0·77	−0·62	−0·55	−0·51	−0·55	−0·59
0·028	−1·30	−0·77	−0·61	−0·50	−0·51	−0·50	−0·48
0·083*	+0·00	−0·35	−0·31	−0·32	−0·40	−0·29	−0·35
0·047*	+0·60	−0·30	−0·32	−0·27	−0·33	−0·27	−0·29

* Some methaemoglobin present.

4. *Preparation of protein solutions and methods of analysis.*

The proteins investigated included the haemoglobins of the sheep and the ox, horse-serum-albumin, total serum-protein, denatured edestin, acid haematin and kathaemoglobin. Some experiments were also carried out on Congo red.

The haemoglobin solutions were prepared by a slight modification of the method given by Adair [1928] which will be described in detail in a later communication. Horse-serum-albumin was recrystallised three times by the method of Adair and Robinson [1930, 1]. The total serum-protein was prepared by the dialysis of untreated serum with phosphate buffer of p_H 7·4. Denatured edestin was made by prolonged dialysis of crystalline edestin, prepared from hempseed, against 0·01 *M* HCl. Acid haematin was obtained by dialysis of haemoglobin against an acetate buffer of p_H 3·04. A soluble form of kathaemoglobin was prepared by mixing equal volumes of 0·9 *M* NaOH and a 22 % solution of haemoglobin (mainly in the form of methaemoglobin) which had been recrystallised by means of ammonium sulphate. After standing at room temperature for a few days, to ensure complete denaturation, the excess of alkali was removed by dialysis, and the alkaline haematin was converted into kathaemoglobin. In some experiments, carbon dioxide was passed through the solution before dialysis.

The Congo red was subjected to a preliminary dialysis against distilled water, in order to remove chlorides, before equilibration with sodium bicarbonate. The concentration of the solution obtained was estimated from dry weight determinations.

In all experiments on protein solutions, the concentrations were determined after equilibration by Kjeldahl nitrogen determinations or by the refractometric method, using factors based on nitrogen determinations [Adair and Robinson, 1930, 1].

The hydrogen ion concentration values of the dialysates at 0° were measured with the Clark hydrogen electrode and a saturated calomel-KCl electrode, standardised by measurements of a mixture of 0·01 *M* HCl in 0·09 *M* KCl. The conventional value $p_H = 2{\cdot}04$ was assigned to this solution. In certain experiments

the p_H value of the protein solution was measured by the hydrogen electrode, but in most experiments it was calculated from the membrane potential and the p_H value of the dialysate.

The following methods were used for analysis of the dialysates: ammonia was estimated by nesslerisation or by the micro-Kjeldahl; phosphates by the method of Bell and Doisy [1920], carbon dioxide according to Van Slyke and Neill [1924], and chlorides by the method of Van Slyke [1923]. The technique for the estimation of sodium will be described in a subsequent communication.

In the analysis of the protein solutions, protein-free filtrates were obtained by heat-coagulation of diluted solutions at the isoelectric point in the presence of sodium sulphate.

In experiments with concentrated buffer solutions (0·05 *M*) complete analyses of the dialysate are not required in all cases, if the refractive indices of the stock buffer and the final dialysate be identical and no difference between their p_H values can be detected by colorimetric methods.

5. Sources of error in measurements of membrane potentials.

The sources of error in measurements of the membrane potentials of protein solutions may include impurities, alterations in the proteins during the process of preparation, deviations from a state of equilibrium between protein solution and dialysate due to the action of bacteria, imperfections in the membranes, changes in the dialysate and breakdown of the protein. During electrometric measurements there are additional sources of error, including alterations in the potentials of the calomel electrodes, contamination of the solutions with potassium chloride, imperfections in the method of forming the liquid junctions and, if the protein solution be not enclosed and maintained at the osmotic equilibrium pressure, there may be errors due to absorption of carbon dioxide, evaporation of water, the dilution of the protein solution and electrokinetic potentials and diffusion potentials which may be correlated with the flow of water across the membrane.

The method described in this work is restricted to solutions that are not greatly affected by carbon dioxide; during the measurements of the potentials, the solutions are exposed to the air. Experiments with buffered solutions including haemoglobin in equilibrium with 0·01 *M* phosphates, in which the solutions were left exposed to the air for several hours, indicated that the amount of carbon dioxide absorbed in the course of an hour was not sufficient to cause appreciable error. In the case of very dilute alkaline solutions, the risk of error due to carbon dioxide is much more serious, and it may be necessary to use an enclosed apparatus of the type figured by Bjerrum [1924].

In the apparatus shown in Fig. 1, the protein solutions are kept under the osmotic equilibrium pressure during dialysis, but the measurements of the potential are made with the protein solution at atmospheric pressure. Under these conditions, the dialysate flows across the membrane into the protein solution. The rate of dilution is not rapid with the type of membrane used in this work, and even if the dismantled membranes are left overnight, the final concentrations usually exceed two-thirds of the initial value. Since the first reading of the membrane potential is made a few minutes after the osmotic pressure is released, the degree of dilution is not appreciable. As a general rule, repetitions of the measurements made within the first hour agreed with the initial value, if the solutions were not contaminated with potassium chloride during the first series of readings. Agreement between the readings made after a time interval shows that the errors due to the fall in protein concentration and to

evaporation of water and absorption of carbon dioxide are not important, but it does not exclude errors due to diffusion potentials which may be associated with the movement of ions of different mobilities across the membrane and electrokinetic potentials due to the flow of fluid across a charged membrane. In neutral solutions collodion membranes are negatively charged, but the charge may be altered by adsorption of protein during equilibration.

Attempts were made to measure these effects in a system containing haemoglobin in equilibrium with 0·01 *M* phosphate buffer, using an apparatus of the enclosed type similar to that figured by Henriques [1928]. It was found that the potentials remained constant within 0·4 millivolt with arbitrary variations of pressure from 6·2 to 140 mm. of mercury.

Since the electrokinetic effects must depend upon the specific properties of the membrane, comparative experiments were set up with collodion, parchment and cellophane membranes. Discs of parchment or cellophane of about 9 cm. diameter were folded over one end of a glass tube 4 cm. long and 1·5 cm. diameter and held in position by rubber rings. The tubes prepared in this way were kept in buffer solution for one or two days; they were then filled, to a depth of about 2·2 cm., with haemoglobin solution which had been subjected to a preliminary dialysis against the same buffer solution. The tubes were placed in small beakers containing glass rods which prevented the parchment or cellophane membranes from touching the bottom of the beakers. The potentials were measured shortly after the tubes were filled with protein solution.

Table II. *Comparison of collodion, cellophane and parchment membranes.*

Exp. 618. Sheep carbon monoxide haemoglobin, 20·28 %. Osmotic pressure 93·6 mm. dialysate 0·01 *M* $NH_4H_2PO_4$ + 0·01 *M* $(NH_4)_2HPO_4$. p_H 7·046 at 0°.

Exp. A 7. Horse-serum-albumin, 15·72 %. Osmotic pressure 38·2 mm. dialysate 0·02 *M* CH_3COONa + 0·01 *M* CH_3COOH. p_H 4·99 at 0°.

Time in minutes. Potentials in millivolts.

618 Collodion		618 Parchment		618 Cellophane		A 7 Collodion		A 7 Parchment	
Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.
0	−1·81	0	−1·86	0	−1·78	0	−4·49	0	−4·25
2	−1·84	1	−1·91	1	−1·82	1	−4·59	2	−4·45
5·5	−1·89	3	−1·96	6	−1·91	5	−4·64	8	−4·70
9	−2·01	9	−2·04			10	−4·84	15	−4·95
15	−2·06	11	−2·06			15	−5·14	27	−5·30
21	−2·16	20	−2·10			18	−5·34	38	−5·35
31	−2·26	36	−2·06						

The results recorded in Table II show that there is a close agreement between the potentials given by different membranes, if comparisons are made at the same interval of time after the formation of the liquid junctions; no evidence is shown for the existence of errors due to specific properties of the membrane. The effects of time on the liquid junction potentials are discussed below.

Information as to the possibility of errors caused by electrokinetic potentials, diffusion potentials and imperfect equilibration may furthermore be obtained by a comparison of the observed membrane potentials with values calculated from electrometric measurements of hydrogen ion activity by a slightly modified form of the equation of Donnan and Allmand [1914].

$$E = 54{\cdot}16 \left(\frac{273+t}{273}\right) (p_{\text{H inner}} - p_{\text{H outer}}) \qquad \ldots\ldots(2).$$

t = temperature in ° C.

This formula has been verified by a number of investigators, including Loeb [1922] and Bjerrum [1924].

The investigations of Hastings *et al.* [1924] and of Stadie and Hawes [1928] indicated that fairly good results might be obtainable with the hydrogen electrode in solutions of carbon monoxide haemoglobin practically free from methaemoglobin. More concentrated solutions, containing 20 to 22 % of protein, used in this work, are very viscous, and the conditions are not ideal for the establishment of equilibrium with hydrogen electrodes at 0°, particularly in the case of haemoglobin. Though consistent results were obtained with most preparations, examples of which are given in Table III, in a long series of experiments the

Table III. *Direct and indirect measurements of membrane potentials of sheep carbon monoxide haemoglobin 20–22 % solutions.*

Dialysates 0·01 *M* ammonium phosphate buffers. E = membrane potential in millivolts.

Exp. no.	Dialysate p_H	Protein solution p_H	Protein solution p_H (calculated)	Direct E	Indirect E	Difference
451	6·295	6·431	6·441	+7·93	+7·3	−0·6
452	6·939	6·942	6·958	+1·03	−0·2	−1·2
454	7·096	7·054	7·089	−0·38	−2·2	−1·8
461	7·680	7·592	7·596	−4·57	−4·77	−0·2
467	6·670	6·706	6·729	+3·17	+2·06	−1·2
469	6·941	6·937	6·953	+0·62	−0·3	−0·9
475	7·398	7·321	7·341	−3·07	−4·15	−1·1
477	8·229	8·087	8·059	−9·20	−7·7	+1·5

hydrogen electrode potentials drifted. In view of the difficulties experienced with haemoglobin, a comparison of membrane potentials, measured by direct and indirect methods, was made on solutions of serum-albumin (Table IV). In

Table IV. *Direct and indirect measurements of membrane potentials of horse-serum-albumin at 0°.*

Exps. A 15, 14, 16, 9, dialysate 0·005 *M* $NH_4H_2PO_4$ + 0·005 *M* $(NH_4)_2HPO_4$.
Exps. A 11, 10, dialysate 0·01 *M* $NH_4H_2PO_4$ + 0·01 *M* $(NH_4)_2HPO_4$.
Exps. A 7, 6, dialysate 0·02 *M* CH_3COONa + 0·01 *M* CH_3COOH.
Exps. A 5, 4, dialysate 0·02 *M* CH_3COONa + 0·025 *M* CH_3COOH.
Osmotic pressures in mm. mercury. E_1 and E_2, initial and final readings of membrane potential in millivolts. E_3 and E_4 potentials calculated from initial and final hydrogen electrode readings.

Exp. no.	Protein g. per 100 ml.	Osmotic pressure	Dialysate p_H	Protein solution p_H	Direct		Indirect	
					E_1	E_2	E_3	E_4
A 15	1·95	7·1	7·056	7·005	− 2·75	− 2·75	− 2·75	− 2·56
A 14	3·36	13·8	7·055	6·963	− 4·47	− 4·54	− 5·05	− 5·05
A 16	5·38	27·3	7·054	6·911	− 6·92	− 7·12	− 7·80	− 7·55
A 9	15·92	171·2	7·041	6·640	−19·0	−20·3	−21·6	−20·2
A 11	15·15	133·6	6·987	6·742	−11·78	−12·58	−12·26	−11·16
A 10	19·09	211·7	7·001	6·709	−14·59	−15·59	−15·8	−15·8
A 7	15·45	38·2	4·985	4·900	− 4·5	− 5·6	− 4·9	− 4·8
A 6	20·06	56·4	4·979	4·867	− 5·38	− 6·68	− 6·1	− 5·9
A 5	15·87	64·6	4·588	4·607	+ 1·2	+ 0·2	+ 1·1	+ 1·0
A 4	19·99	91·2	4·582	4·603	+ 1·47	+ 0·33	+ 1·1	+ 1·7

dilute solutions of serum-albumin, there is a satisfactory agreement between the potentials determined by both methods. In solutions containing more than 15 % of the protein the experimental difficulties were greater on account of the effects at the liquid junctions, discussed below. The average discrepancy between

the initial readings of observed and calculated potentials was 0·6 millivolt, which corresponds to 0·01 p_H unit. The maximum discrepancy was 1·4 millivolts.

Traces of carbon dioxide, absorbed during dialysis, may cause small errors in indirect determinations of membrane potentials by means of hydrogen electrode measurements, because varying amounts of carbon dioxide may be removed by bubbling hydrogen through the liquids. The p_H changes due to loss of carbon dioxide are not identical in the protein solution and in the dialysate on account of the buffer action of the proteins. The agreement between the direct and indirect determinations, shown in Tables III and IV, is evidence against the existence of serious errors due to carbon dioxide.

The magnitude of the experimental errors due to liquid junction potentials depends upon the composition of the solutions under examination. The reproducibility of the liquid junctions formed in the electrodes illustrated in Fig. 1 was tested by measuring the potential difference between two electrodes dipping into the same solution. In the case of dilute buffer solutions the potentials agreed, but in concentrated (M) ammonium phosphate buffers there were variations of 0·5 millivolt. If the initial measurements were reproducible, the constancy of the potentials could be investigated by renewing the liquid junction in one of the electrodes at known intervals of time. In 0·01 M ammonium phosphate buffers, where it is possible to obtain initial values agreeing within 0·03 millivolt, the potential of the electrode left undisturbed in the solution remained constant within 0·03 millivolt for 30 minutes. In dilute solutions of proteins equilibrated with 0·01 to 0·05 M buffer solutions, the initial readings were reproducible but the variations with time might exceed 0·1 millivolt.

In solutions containing 20 % of haemoglobin, the results were reproducible if both junctions were formed within 30 seconds, but a freshly-formed junction was more positive than one left standing for a few minutes. The differences ranged from 0·2 to 0·5 millivolt, in cases where the first junction had been left standing for 15 minutes. In concentrated solutions of serum-albumin, the initial readings agreed, but the difference between a freshly formed and an older junction ranged from 1·0 to 1·3 millivolts.

Comparisons made between electrodes with wide and narrow tips showed little difference in the case of buffers and of haemoglobin solutions, but there were differences ranging from 0·2 to 0·6 millivolt in the case of serum-albumin.

It is possible that these variations with time are correlated with an alteration at the boundary between solution and saturated potassium chloride, which has been observed not only with this type of electrode but with liquid junctions formed by means of taps. At first the boundary is sharp, but within a few minutes it becomes irregular. The disturbance is probably due to convection currents, because the mixture formed by the diffusion of potassium chloride into the protein solution may be denser than the pure potassium chloride solution below it.

In a measurement of the membrane potential, the observed potential E can be regarded as the sum of E (KCl.Pr), the liquid junction potential at the boundary between saturated potassium chloride and protein solution, E_m, the potential at the membrane and E (D.KCl), the potential at the junction of the dialysate and the potassium chloride solution of the second electrode. The observations recorded in Table II show that the final values of E are more negative than the initial values, and the direction and magnitude of the changes are in accordance with those observed in studies of the liquid junction E (KCl.Pr), a result which might be expected if the potentials E_m and E (D.KCl) remained constant.

Observations of the potentials at different times after the formation of the liquid junctions are furthermore of value in detecting errors due to contamination of the solutions with potassium chloride, which may cause drifts in the potentials which are larger and more rapid than those due to liquid junction potentials. Rapid drifts may also be caused by local differences in the concentration of ions due to imperfect equilibration. It is advisable to reject experiments which show drifts in the potential which cannot be accounted for by liquid junction potentials. With dilute solutions of proteins, the potentials should remain constant within 0·1 millivolt.

In many problems, the accuracy of the ratio E/C, where C is the protein concentration, is more important than the accuracy of the potential E, and therefore an error of 0·5 millivolt on a 25 % solution is comparable with an error of 0·02 millivolt on a 1 % solution.

With the exception of the experiments in Tables II and IV, which exemplify the maximum variations with time, the potential values recorded graphically and in the Tables are those obtained between 3 and 4 minutes after the formation of the liquid junction in the protein solution. The practice of recording initial readings in measurements on inorganic solutions was adopted by Lewis *et al.* [1917] and by Clark [1928]. According to Maclagan [1929] and Guggenheim [1930], the final readings, obtained 10 or 15 minutes after the formation of the liquid junction, are more reproducible in the case of junctions formed with hydrochloric acid inside tubes.

In certain of our experiments, the final as well as the initial readings were reproducible, but it is more difficult to obtain satisfactory final readings on account of contamination of the solutions with potassium chloride. It is possible that in the case of protein solutions the initial readings represent a closer approximation to a sharp junction, and it is by no means certain that the final readings are of greater significance.

As the initial readings of membrane potentials that we have obtained for solutions of different protein concentration are reproducible, correction factors could be determined if a standard type of liquid junction for protein solutions were to be devised at a future date.

Henriques [1929], who used an electrometer, drew attention to polarisation as a possible source of error in measurements of membrane potentials by the use of a galvanometer and a potentiometer. In this work it was found that there was no difference between the potentials measured just before and immediately after attempts to polarise the system by closing the circuit with the potentiometer set at 5 or 10 millivolts above or below the equilibrium value, causing galvanometer deflections from 57 to 112 mm.

6. *Measurements of membrane potentials at different protein concentrations in solutions where the composition of the dialysate is kept constant.*

The observations recorded in Figs. 2 to 6 show that in all the protein systems investigated, which included proteins such as haemoglobin and serum-albumin with particles of uniform size and denatured proteins which may be mixtures of particles of different sizes, there is a straight line relationship between the membrane potential and the "corrected percentage concentration," C_g, defined by formula 3, under conditions in which the membrane potential is small.

The results of analyses of the protein solutions by volumetric or by refractometric methods are usually expressed in terms of C, the concentration of dry

protein in g. per 100 ml. of solution, and the values of C_v are calculated from these measurements.

$$C_v = C\,[100/(100 - V_1 C)] \qquad \ldots\ldots(3).$$

C_v = g. of dry protein per 100 ml. of solvent. C = g. of dry protein per 100 ml. of solution. V_1 = volume occupied by 1 g. of dry protein.

The volume of V_1 cannot be less than 0·75 ml., the specific volume of the dry protein, and it may be more if the protein combines with water.

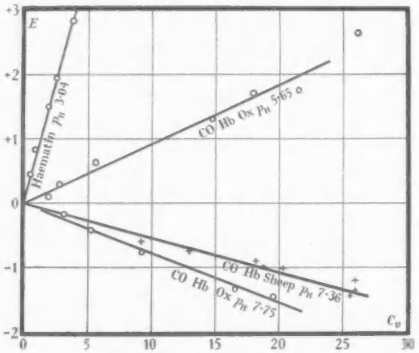

Fig. 2. Membrane potentials of CO-haemoglobin and acid haematin at different concentrations.

Dialysates of ionic strength 0·01, compositions given in mols. per litre:

p_H 3·04. CH_3COONa 0·0025, CH_3COOH 0·0975, NaCl 0·0975.
p_H 5·65. CH_3COONa 0·100, CH_3COOH 0·0105.
p_H 7·36. $(NH_4)_2HPO_4$ 0·03, $NH_4H_2PO_4$ 0·01.
p_H 7·75. Na_2HPO_4 0·0321, KH_2PO_4 0·00357.

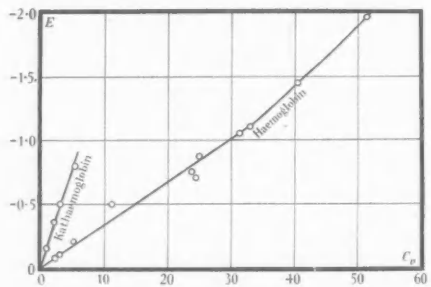

Fig. 3. Membrane potentials of haemoglobin (mixture of oxygenated and reduced) and kathaemoglobin from sheep blood.

Dialysate p_H 7·8, ionic strength 0·289, KCl 0·1, Na_2HPO_4 0·0613, KH_2PO_4 0·00533.

The hydration of haemoglobin, estimated from chloride distribution measurements, is 0·21 g. per g. of dry protein [Adair, 1928]. The hydration of the haemoglobin of the horse in the presence of ammonium sulphate has been investigated by Sørensen and Sørensen [1933]. The value 0·353 g. given in their paper is reduced to 0·306 g. if the calculation is based on the nitrogen determination of 16·69 %, made by Vickery and Leavenworth [1928], rather than the

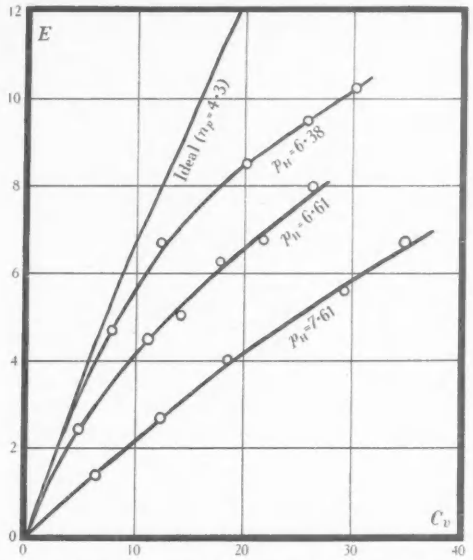

Fig. 4. Membrane potentials of sheep CO-haemoglobin.

"Ideal" n_p=4·3 curve calculated for a protein of valence 4·3 in ideal solutions.
Dialysates of ionic strength approximately 0·01.

p_H 6·38. $(NH_4)_2HPO_4$ 0·00128, $NH_4H_2PO_4$ 0·00672.
p_H 6·61. $(NH_4)_2HPO_4$ 0·0017. $NH_4H_2PO_4$ 0·0053.
p_H 7·61. $(NH_4)_2HPO_4$ 0·0037. $NH_4H_2PO_4$ 0·0012.

At p_H 7·61 potentials are negative.

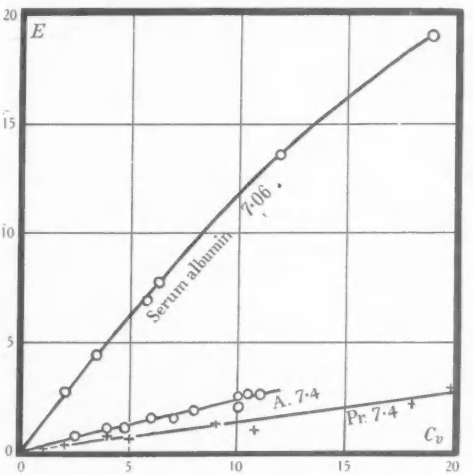

Fig. 5. Membrane potentials of serum-proteins.

Serum-albumin, p_H 7·06. Dialysate $(NH_4)_2HPO_4$ 0·005, $NH_4H_2PO_4$ 0·005. A 7·4 serum-albumin, Pr 7·4 total protein. Dialysate p_H 7·4. Na_2HPO_4 0·0533, KH_2PO_4 0·0133.

older value of 17·3 %. Adair and Robinson [1931] used a direct method, in which the dried haemoglobin of the sheep, exposed to the vapour of saturated ammonium sulphate solution, took up 0·2 g. of water per g. dried protein. Weber and Nachmannsohn [1929] estimated that the effective volume $V_1 = 1{\cdot}0$ ml. in the case of serum-albumin. In the calculations given below, the volume $V_1 = 1{\cdot}0$ has been used for serum-albumin and $V_1 = 0{\cdot}965$ for haemoglobin and its derivatives. Although these values may require correction when more complete

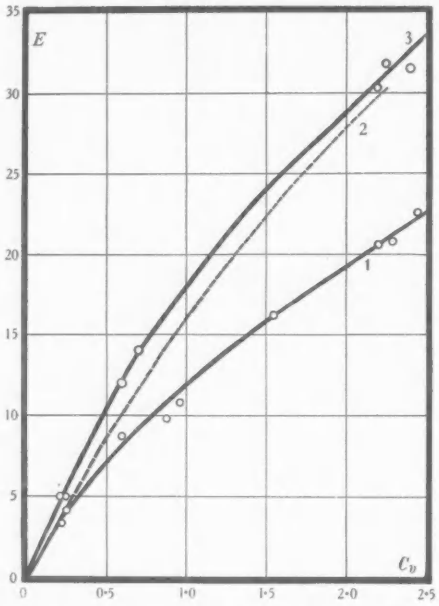

Fig. 6. Membrane potentials of edestin. Dialysate 0·0104 *M* HCl. Curve 1, observed potentials. Curve 2, theoretical potentials for protein of constant valence 26·3. Curve 3, potentials calculated from chloride concentrations (Section 8).

data are available, the straight line relationships between the membrane potentials and C_p shown in Fig. 2 indicate that the corrections are small, although it is not impossible that a straight line might be obtained in a system where the error due to an underestimate of the hydration value is compensated by a reduction in the membrane potentials in concentrated solutions, due to the interionic forces described by Debye and Hückel [1923].

7. *Relationships between membrane potentials and protein concentrations in ideal solutions.*

Bjerrum [1924] showed that in an infinitely dilute solution of a colloid equilibrated with hydrochloric acid, the potential is proportional to the concentration of the colloid in mols. per litre of solution.

The calculations given below show that in ideal solutions the potentials are proportional to the concentrations in g. mols. per litre of solvent, under certain

conditions which can be defined by the application of the formula (4), due to Donnan and Guggenheim [1932], together with the additional calculations recorded in Table V.

$$(P'-P'')\,v_i\left(1-\kappa_i\frac{P'+P''}{2}\right)+RT\,ln\frac{N_i'}{N_i''}=\frac{-n_iFE}{1000} \qquad \text{.....(4).}$$

R, T, F and E are defined as for formula (1). v_i = the volume of the ion in litres per g. mol. κ_i = the compressibility of the ion. n_i = the valence (negative for anions). N_i' = the molar fraction of the ion in the protein solution. P' = the hydrostatic pressure of the protein solution. N_i'' = the molar fraction of the ion in the dialysate. P'' = the hydrostatic pressure of the dialysate.

In all the formulae given below, where it is necessary to distinguish between the protein solution and the dialysate, the same notation is used. The symbols with two accents refer to the dialysate.

The effects of hydrostatic pressure, discussed by Hückel [1925] and represented by the first term in formula (4), are very small in colloidal solutions with low osmotic pressures. According to Kameyama [1925], a pressure of one atmosphere is equivalent to a correction of 0·06 % in the distribution of potassium chloride across a membrane, a correction which can be neglected in comparison with the experimental errors in the measurements of the molar fraction of the ions.

In aqueous solutions, the absolute values of the molar fractions are uncertain, on account of the polymerisation of water, but it is possible to calculate conventional values by formula (5), assuming that 1000 g. of water equal 55·51 mols. [Lewis and Randall, 1923].

$$N_i'=x_i'/(55{\cdot}51+S') \qquad \text{.....(5).}$$

x_i' = the molality of the ion in g. mols. per 1000 g. of water in the protein solution. S' = the sum of the molalities of all solutes including the protein present in the solution.

A similar formula $N_i''=x_i''/(55{\cdot}51+S'')$ can be applied to the dialysates. The sum of the molalities S'' is less than S', because the dialysate does not contain protein.

If, however, the difference between S' and S'' is of the order of 0·01 g. mol. per 1000 g. of water, the denominators in the formulae differ by less than 0·03 % and it follows that formula (4) is equivalent to the simpler formula:

$$x_i'\text{ (ideal)}=x_i''r_i \qquad \text{.....(6).}$$

r_i = the ideal distribution ratio, defined by formula (7).

$$r_i=\text{antilog}\,(-2{\cdot}303n_iFE/1000RT) \qquad \text{.....(7).}$$

At 0°, $2{\cdot}303\,n_iFE/1000\,RT=n_iE/54{\cdot}16$.

The conditions under which formula (6) is valid can be determined from measurements of the osmotic pressures of the solutions with collodion membranes impermeable by proteins. Sørensen [1917] pointed out that the osmotic pressure of an ideal solution of a protein, in which the molar fraction of the protein is small, is determined by formula (8).

$$p\text{ (ideal)}=RTm_p \qquad \text{.....(8).}$$

m_p is the corrected concentration of the protein, expressed in g. mols. per litre of dispersion medium. If p (ideal) be expressed in mm. mercury at 0°, then RT, expressed in mm. mercury and litres, is equal to $760\times22{\cdot}4=17{,}033$ at 0°. In dilute aqueous solutions at 0°, there is little difference between the corrected concentration and the molality, and if the osmotic pressure be below 170 mm. mercury, the difference between S' and S'' cannot exceed 0·01.

It is convenient to use the same units in calculations of osmotic pressures and of the distribution of ions, namely, the corrected concentrations, which are correlated with concentrations and molalities by formula (9).

$$m_i' = c_i' \,(100/100 - V_1 C) = x_i'/w' \qquad \text{.....(9).}$$

m_i' = the corrected concentration of the ion in g. mols. per litre of solvent in the protein solution. c_i' = g. mols. per litre of protein solution. V_1 and C = effective volume and concentration of protein (formula (3)). w' = kg. of water per litre of solvent.

In the dialysate, the volume of solvent, defined above, is equal to the volume of the solution, and therefore $m_i'' = c_i'' = x_i''/w''$, where w'' is the water content of the dialysate. The terms w'' and w' are almost identical in a system where the molar fraction of water in the protein solution is 99·98 % of its value in the dialysate, a value corresponding to an osmotic pressure of about 170 mm. mercury, and therefore formula (10) can be applied to solutions with low osmotic pressures.

$$m_i' \text{ (ideal)} = m_i'' r_i = c_i'' r_i \qquad \text{.....(10).}$$

Further steps in the calculation of the relationship between the membrane potential and the protein concentration are shown in Table V.

Table V. *Relationship in ideal solutions between membrane potentials and the equivalent concentration of the protein, equilibrated with salts of different valence types.*

Type (1–1). Example Pr.Cl + HCl. Type (2–1). Example Pr.HPO_4 + Na_2HPO_4. Type (1–2). Example NaPr + Na_2HPO_4, potentials negative. Type (1–1·5). Example NaPr + Na_2HPO_4 + NaH_2PO_4. $\Delta p_H = p_H$ difference between protein solution and dialysate.

E	Δp_H	$\frac{[Cl]_c'}{[Cl]''}$	$\frac{[H]_c'}{[H]''}$	u	Type (1–1) $\frac{m_p n_p}{J}$	Type (2–1) $\frac{m_p n_p}{J}$	Type (1–2) $\frac{m_p n_p}{J}$	Type (1–1·5) $\frac{m_p n_p}{J}$
1·18	0·022	1·051	0·951	0·05	0·050	0·051	0·049	0·049
2·35	0·043	1·105	0·905	0·10	0·100	0·105	0·095	0·096
4·72	0·087	1·221	0·819	0·20	0·201	0·224	0·184	0·188
7·05	0·130	1·350	0·741	0·30	0·305	0·360	0·267	0·276
9·40	0·174	1·492	0·670	0·40	0·411	0·518	0·348	0·363
11·75	0·215	1·649	0·606	0·50	0·521	0·704	0·427	0·450
16·45	0·304	2·014	0·497	0·70	0·759	1·695	0·589	0·631
23·54	0·434	2·718	0·368	1·00	1·175	2·340	0·861	0·940

In making these calculations, it is assumed that the experimental data given are the membrane potentials and the concentrations of the ions in the dialysate, but as all the terms given in the tables are functions of the membrane potentials, there are many alternative sets of data that could be utilised. The first column gives the membrane potentials in millivolts at 0°, the temperature at which the measurements were made in this work. For comparison with Loeb's measurements at 24°, the potentials should be multiplied by (273 + 24)/273. The terms in the other columns require no temperature corrections. The p_H difference between protein and dialysate is recorded in the second column. The third column gives the ideal distribution ratio for chloride ions, which may be calculated by formula (10) or by the exponential formula stated below:

$$r_i = e^{-n_i u} = [1 - n_i u + \tfrac{1}{2} n_i^2 u^2 - \tfrac{1}{6} n_i^3 u^3 + \ldots] \qquad \text{.....(11).}$$

$u = E \times F/1000RT = E/23{\cdot}535$ at 0°.

The fourth column gives the ideal distribution ratio for the hydrogen ions. Since the concentrations of the ions in the dialysate, symbolised $[H]''$ and $[Cl]''$, are known, their corrected concentrations in the protein solution, symbolised $[H]_c'$ and $[Cl]_c'$, can be calculated from the distribution ratios. The equivalent concentration of the protein chloride is then calculated as the difference between $[Cl]_c'$ and $[H]_c'$, and if m_p, the corrected concentration of the protein ions, is known, the valence, or more precisely the mean valence of the protein ions, defined as the ratio of the equivalent and the molar concentrations, can then be calculated by formula (12).

$$m_p n_p = [Cl]_c' - [H]_c' \qquad \text{.....(12).}$$

If the exponential series in formula (11) is applied, the term $[Cl]_c'$ is equal to $[Cl]''(1+u+\frac{1}{2}u^2)$, and $[H]_c'$ equals $[H]''(1-u+\frac{1}{2}u^2)$ and if u be small, so that higher powers of u can be neglected, $m_p n_p$ is equal to $u([Cl]''+[H]'')$.

Formula (12) differs from the equations given by Bjerrum [1924] and by Adair [1928] in that c_p is replaced by m_p. Similar calculations show that if the dialysate contains a mixture of ions of different valences, $m_p n_p$ is determined by formula (13).

$$m_p n_p = -\Sigma m_i' n_i \qquad \text{.....(13).}$$

$\Sigma m_i' n_i$ = the sum of the corrected concentrations of the ions in the protein solution, multiplied by their valences. The ideal values of m_i' are determined by formula (10). If u is small, formula (14) can be applied:

$$m_p n_p = uJ \qquad \text{.....(14).}$$

J = the sum of the concentrations of the ions in g. mols. per litre of dialysate, multiplied by the squares of their valences. Both n_p and u are negative when the protein is acting as an anion.

In Table V, exact values of the ratio $m_p n_p/J$ have been calculated, not only for univalent ions but also for mixtures such as the phosphate buffers containing bivalent ions, by the application of formulae (13) and (10), using tables of values of e^u and e^{-u} to calculate the distribution ratios referred to in formula (11). The simpler formula (14) can be applied over the range where the difference between u and $m_p n_p/J$ is small.

8. *The determination of the valence of protein ions.*

Pauli and Valkó [1933] have discussed the difficulties inherent in estimations of the valence of protein ions and have given references to previous investigations bearing on this problem. The method of cataphoresis is of historical interest, because the existence of a charge on protein ions, which depends upon the hydrogen ion concentration, was first demonstrated by measurements of the mobilities of the proteins in an electric field [Hardy, 1899; 1905].

The calculation of the charge presents considerable difficulties, on account of the uncertain effects of the "atmosphere" of inorganic ions surrounding the colloidal particle, referred to by Tiselius [1930], who has made most accurate measurements of the mobilities of proteins, and by Abramson [1933].

All other methods depend upon estimates of the concentrations of the inorganic ions present in the free state in the protein solution and the application of a formula of the type stated below:

$c_p n_p$ = sum of equivalents anions − sum of equivalents cations

.....(15).

The concentrations of the ions in the free state cannot be determined directly; the results obtained by chemical analyses represent the sum of free and combined ions; the activities of the ions measured by electrometric methods may be different from the true concentrations, unless the solutions are so dilute that it is possible to neglect the interionic forces described by Debye and Hückel [1923]. Similar difficulties arise in estimates based upon osmotic pressures, conductivities and membrane potentials of the solutions.

The method described in this work is based on measurements of membrane potential, but it differs in certain respects from the methods described by Bjerrum [1924], Rinde [1926] and other investigators. Firstly, the measurements are made on solutions with relatively small membrane potentials and secondly, the relationship between the potential and the protein concentration is determined under conditions where the composition of the dialysate is kept constant, as shown in Figs. 2 to 6.

In the theoretical interpretation of the curves, it must be remembered that the pure salt solutions of the dialysates do not follow the ideal solution laws. The formula of Donnan and Allmand, discussed in Section 1, for membrane potentials in non-ideal solutions, can be stated in the form $a_i' = a_i'' r_i$, where a_i' and a_i'' denote the activities of the ion in the protein solution and in the dialysate, or in the alternative form $x_i' f_i' = x_i'' f_i'' r_i$, where f_i' and f_i'' denote the activity coefficients of the ions; it follows that $m_i' (f_i'/f_i'') = m_i'' r_i$ (formula (16)) within the range of application of formulae (6) and (10).

According to the theory of Debye and Hückel [1923], the activity coefficient is a function of the ionic strength of the solution, which is equal to $\frac{1}{2}J$. The calculations recorded in Table V show that in systems with large membrane potentials, there is a marked excess of ions inside the membrane. The difference between the values of J' and J'' in the protein solution and in the dialysate must affect the activity coefficients. If the membrane potential be less than 2 millivolts, the excess of ions inside the membrane is relatively small, and unless there are specific effects due to the protein, the ration f_i'/f_i'' should approach unity. If this ratio be equal to unity at very low protein concentrations, formula (16) is the same as formula (10), and it follows that the straight line relationship shown by formula (14) and by Table V can be applied to the non-ideal as well as the ideal solution. The valence of the protein ions is then determined by formula (17).

$$n_p = \left(\frac{uJ}{m_p}\right)_0 = 0{\cdot}00425\, M.J \left(\frac{E}{C_v}\right)_0 \qquad \text{.....(17).}$$

0·00425 is calculated for a temperature of 0° using the constants in formula (11). M = the molecular weight of the protein. $(E/C_v)_0$ = the value of the ratio (E/C_v) at $C_v = 0$, estimated by extrapolation from a series of measurements in which the composition of the dialysate is kept constant. $C_v = m_p.M/10$.

An experimental test of the formula (17) has been made by determining the relationships between E and C_v for a number of different proteins and a number of different solvents, covering a p_H range from 2·0 to 7·8 and ionic strengths from 0·01 to 0·287. In all the systems investigated, there is a straight line relationship between E and C_v over a range from 0 to 2 millivolts, as shown in Figs. 2 to 6, a result in accordance with the assumptions made in the derivation of formula (17).

Deviations from the ideal relationships are observed only in solutions where the membrane potentials are large as in the case of haemoglobin phosphate (Fig. 4) and edestin chloride (Fig. 6), but under these conditions there is a marked excess of ions inside the membrane which must alter the activity coefficients.

A part of the difference between the ideal and the observed curves for haemoglobin phosphate can be accounted for by the difference between the p_H values of the protein solution and the dialysate and the effects of the p_H on the valence, a subject which will be discussed in a subsequent communication.

Although the results recorded in Figs. 2 to 6 are all consistent with formula (17) and show that n_p is constant in solutions with low membrane potentials, they do not prove that the absolute value of n_p is the same as that calculated by formula (17). Evidence bearing on the absolute value of n_p is given below, but the results cannot be regarded as decisive because all alternative methods are subject to uncertainty.

Congo red and 0·02 M *$NaHCO_3$*. The determination of the valence of Congo red from measurements of membrane potential is of interest because in the case of this colloid, independent evidence concerning the degree of ionisation has been obtained by osmotic methods. Bayliss [1911] and Donnan and Harris [1911] obtained osmotic pressures about one-third of those calculated for a fully ionised disodium salt Na_2R, and Donnan and Harris showed that the observed pressures could be accounted for if Congo red ionised according to the equation

$$(Na_2R)_n = n\,Na + (NaR)_n,$$

where n is the number of molecules present in the colloidal micelle of Congo red. The electrical conductivities indicate greater ionisation, but this difference may be accounted for by McBain's theory that the ionic micelles have a relatively high mobility [McBain *et al.*, 1919].

The data recorded in Table VI show that the equivalent concentration $m_p n_p$, deduced from the membrane potentials, is from 47 to 53 % of the total sodium equivalent to Congo red, a result in accordance with the osmotic measurements of Donnan and Harris.

Table VI. *Membrane equilibrium of Congo red at 0°. Dialysate 0·02* M *$Na_2CO_3 + NaHCO_3$. p_H 9.*

All concentrations expressed in millimols. per litre, except that of Congo red, given in milliequivalents.

$[CO_2]''$ = sum of $[HCO_3]'' + [CO_3]''$. Proportions of ions determined by formula $[Na]'' = (1 + x'')[CO_2]$, where x'' = fraction of total CO_2 present as carbonate in the dialysate. $[CO_2]'$ = total CO_2 in the colloidal solution. $[CO_2]_c'$ (calc.) computed from values of $[CO_2]''$, x'' and E by application of formula $m_i' = m_i'' r_i$. $-m_p n_p$ = corrected equivalent concentration of Congo red computed from formula $-m_p n_p = [Na]_i'$ (ideal) $- (1 + x')[CO_2]$ (ideal). x' = fraction of total CO_2 present as carbonate. Values for [Congo red] represent total sodium equivalent to Congo red, determined by analyses of $[Na]'$ and $[CO_2]'$ and the calculation of x'. Observed values of [Congo red] $[Na]'$ and $[CO_2]'$ uncorrected because of the low concentrations of Congo red (0·51 to 1·79 g. per 100 ml.).

Dialysate		Inner fluid					
$[Na]''$	$[CO_2]''$	[Congo red]	$[Na]'$	$[CO_2]'$	$[CO_2]_c'$ calc.	$-m_p n_p$	E
20·2	18·9	14·4	30·8	15·5	15·6	7·7	− 4·21
20·1	18·9	22·0	37·1	14·4	14·5	10·5	− 5·87
19·9	19·0	24·0	38·4	13·9	14·3	11·3	− 6·42
19·9	19·0	25·9	40·1	13·8	13·5	13·1	− 6·92
20·2	18·9	32·8	45·8	12·4	12·6	16·4	− 9·04
20·2	19·2	50·3	61·3	10·7	10·8	23·7	−12·92
20·2	19·2	51·0	61·7	10·4	10·6	24·8	−13·48

Edestin chloride and 0·0104 M *HCl*. The ratio $(E/C_v)_0$, calculated from the experiments on edestin recorded in Fig. 6, is equal to 17·5, and making the provisional assumption that in a solution of edestin denatured by hydrochloric

acid, the mean molecular weight is 17,000, the mean valence is 26·3. An approximate confirmation of this value is afforded by the determinations of the chloride concentrations on both sides of the membrane by chemical analyses. The curve marked 3 in Fig. 6 gives the potentials calculated from these analyses by the application of formulae (7) and (10) on the assumption that the protein chloride is fully ionised. Even in the most concentrated solutions, the observed potentials are about 70 % of the calculated values.

A rough estimate of the valence of edestin as 24·0 has been made on the assumptions that the chloride is fully ionised and that the activity coefficient of the hydrogen ions is the same as that of the chloride ions [Adair, 1928]. The electrometric measurements of Hitchcock [1923] on an edestin preparation not exposed to prolonged dialysis with acid give the slightly smaller value of 21. The criticism published by Stadie and Sunderman [1931] of the value 24·0 is based on a misunderstanding. They overlook the fact that in solutions of protein chlorides equilibrated with free hydrochloric acid, it is necessary to deduct the free hydrochloric acid from the total chloride in order to estimate the chloride equivalent to the protein. In their criticism of Adair's conclusion that an increase in the concentration of edestin diminishes the activity coefficient of the chloride ions, they point out that the electrometric measurements involved liquid junction potentials. It is unlikely that the change in the activity coefficient can be accounted for by the liquid junction potentials, because the protein diminishes not only the activity coefficients but also the osmotic coefficient g' defined by the formula $\pi = RTg'$ ([H]+[Cl]+[Pr]), where π is the total osmotic pressure. In solutions containing 0·246, 0·60 and 2·16 g. of protein per 100 ml. the values of g' were 0·94, 0·85 and 0·73 respectively.

Serum-albumin. The potentials of serum-albumin recorded in Table VIII are relatively large, and instead of determining the ratio $(E/C_v)_0$ by extrapolation, the "apparent valence" at each protein concentration was calculated, as shown in Table VIII. The limiting value for $C_v=0$, determined by extrapolation, is $-17{\cdot}1$. The apparent valences diminish as the protein concentration increases, but in all cases they exceed the values of y, the number of ammonium ions equivalent to the protein determined by chemical methods. The values of y are subject to error on account of the technical difficulties of the analyses, but the difference between n_p and y exceeds the experimental errors.

In a pure aqueous solution of an ammonium proteinate, the value of n_p cannot be greater than y, and it may be less if the proteinate be not fully ionised. In the presence of neutral salts, it is theoretically possible for n_p to exceed y, if the protein exists as a zwitterion, with many positive and negative charges, and if the anions of the neutral salt are capable of combining with, or neutralising some of the positive charges of the zwitterion. In the case of a zwitterion, n_p represents the difference between the numbers of positive and negative charges, and it is possible to increase n_p without altering the amount of acid or base combined, as determined by titration curves and by analyses of the type recorded in Table VII. This hypothesis is open to objection on chemical grounds, because the salts of ammonia and its simpler derivatives appear to belong to the class of strong electrolytes in aqueous solutions, and therefore a compound of the type $R\,NH_3Cl$ should be fully ionised [Sidgwick, 1927]. The evidence is not decisive, because a protein may have different types of positive groups.

If unionised phosphate be present associated with the protein, the observed concentration of the phosphates (m_i') should exceed the ideal value.

$$m_i' \text{ (observed)} = m_i'' r_i + b m_p \qquad \text{.....(18).}$$

The term b represents the apparent combination of ions with the protein, the sum of b_1, the number of ions combined with one molecule of the protein and of b_2, which represents the excess of ions in the neighbourhood of the protein which may be attracted by the interionic forces. Formula (18) differs from an equation published by Northrop and Kunitz [1926] in that a correction for the hydration of the protein is made in the calculation of m_i' by formula (18), and that b is regarded as the sum of two terms and is expressed in corrected concentrations rather than molalities.

The observations recorded in Table VII showed differences between the ideal and the observed concentrations of ammonium ions scarcely greater than the experimental errors. Theoretically, the ammonium proteinate tends to diminish the activity coefficients, but the relatively low concentration of the bivalent ion HPO_4 in the protein solution acts in the opposite direction. The observed concentrations of total phosphate are greater than the ideal concentrations, and the values of b recorded in Table VIII show an excess of about four phosphate molecules per molecule of albumin in dilute solutions, which is sufficient to account for the difference between y and n_p, on the assumption that the phosphate is unionised. The estimate of b, the apparent combination of phosphate ions with the protein, is affected by errors in the electrometric measurements as emphasised by Stadie and Sunderman [1931], but the chemical measurements, given in Table VII, show that the product of the corrected con-

Table VII. *Membrane equilibrium at 0° of serum-albumin and 0·005* M $NH_4H_2PO_4 + 0{\cdot}005$ M $(NH_4)_2HPO_4$.

Mean p_H of dialysates after equilibration = 7·06. All concentrations in millimols. per litre solvent. m_p = corrected concentration of albumin, assuming mol. wt. 72,000. $[PO_4]''$ = total phosphate in dialysate = $HPO_4 + H_2PO_4$. x' = fraction of total phosphate in protein solution in bivalent form, computed from $[NH_4]''$, $[PO_4]''$ and E.

m_p	$[NH_4]''$	$[NH_4]_c'$ ideal	$[NH_4]_c'$ observed	$[PO_4]''$	$[PO_4]_c'$ ideal	$[PO_4]_c'$ observed	x'
0·287	15·11	17·00	17·50	10·07	8·47	9·71	0·471
0·482	14·95	18·08	18·50	9·73	7·31	9·61	0·489
0·792	14·80	19·85	20·6	10·15	6·70	9·73	0·386
0·882	15·15	21·11	20·3	10·17	6·30	9·15	0·408
1·645	14·75	26·20	27·9	10·00	4·46	8·86	0·339
3·320	15·00	41·20	37·4	10·00	2·48	9·15	0·267
2·480*	29·90	49·20	48·6	20·40	10·12	19·78	0·346
3·280*	29·97	55·70	48·9	20·00	8·28	19·80	0·350

* 0·02 *M* phosphate buffer.

Table VIII. *Observed membrane potentials of serum-albumin and ammonium phosphate at 0°.*

n_p, the apparent valence, computed from data in Table VI by formula $-m_p n_p = [NH_4]_c'$ (ideal) $-(1+x')[PO_4]_c'$ (ideal). y = base per mol. protein, computed from formula $m_p y = [NH_4]_c'$ (observed) $-(1+x')[PO_4]_c'$ (observed). b = excess PO_4 per mol. protein, defined by formula (18). p = osmotic pressure in mm. mercury. p_H represents the protein solution.

m_p	E	p	p_H	n_p apparent	y	b
0·287	− 2·75	7·1	7·00	− 15·8	11·2	4·3
0·482	− 4·47	13·8	6·96	− 14·9	8·7	4·8
0·792	− 6·92	27·3	6·91	− 13·4	9·0	3·8
0·882	− 7·78	32·5	6·88	− 13·9	8·4	3·2
1·645	− 13·56	90·7	6·80	− 12·3	9·9	2·6
3·320	− 23·80	251·8	6·64	− 11·5	8·5	2·0
2·480	− 11·78	133·6	6·74	− 14·3	8·9	3·9
3·280	− 14·59	211·7	6·71	− 13·5	6·8	3·5

centrations of the ions is considerably greater in the protein solutions than in the dialysates, and that the protein must affect the mean activities of the ions of ammonium phosphates, although such measurements do not prove that the phosphates specifically are affected by the protein.

In addition to the measurements on ammonium phosphates, a number of experiments were made with a buffer containing 0·0533 mol. of Na_2HPO_4 *plus* 0·0133 mol. of KH_2PO_4, p_H 7·4 at 0°, which showed that the provisional estimate of $(E/C_v)_0=0·32$, given by Adair and Robinson [1930, 2], is too large. The value of $n_p=-25·4$ has been calculated from a more recent determination $(E/C_v)_0=0·24$. This value of 25·4 is larger than that obtained from 0·01 *M* ammonium phosphates at p_H 7·06 (17·1), but the difference is accounted for by the composition of the buffers.

Although the results obtained with serum-albumin and phosphate mixtures are consistent with formula (17) if the additional assumption is made that the phosphate is not completely ionised, they cannot be regarded as a proof of the formula on account of the lack of independent evidence.

Summary.

The sources of error in measurements of the membrane potentials of protein solutions have been investigated, and a method has been developed which can be applied to systems where the potentials are small and volumes of the solution available are restricted to 5 ml. or 1·5 ml.

The haemoglobin of sheep and ox, serum-albumin, acid haematin, kathaemoglobin and edestin have been equilibrated at 0° with dialysates of well-defined hydrogen ion concentration, with p_H values ranging from 2·0 to 7·8 and salt concentrations from 0·005 to 0·16 *M*, and it has been found that the observed relationship between the membrane potential E and the concentration C_v, expressed in g. protein per 100 ml. solvent, is a close approximation to a straight line when E is less than 2·0 millivolts.

The hypothesis is advanced that, from such measurements, it is possible to calculate the mean valence of the protein ions, symbolised n_p, by the formula $n_p=0·00425\,MJ\,(E/C_v)_0$, where M is the molecular weight of the protein and J the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valences. $(E/C_v)_0$ is the limiting value of the ratio at $C_v=0$, determined by extrapolation. The hypothesis is consistent with measurements of the relationships between membrane potentials and protein concentrations and with additional evidence obtained from chemical analyses and osmotic pressure determinations on dilute solutions of edestin and on Congo red.

In the case of serum-albumin, the results obtained can be reconciled with the investigations of the distribution of ions on the assumption that the protein exists as a zwitterion and that some of the positive charges are neutralised by phosphate ions.

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XXX. THE ACIDS PRODUCED FROM SUGAR BY A *PENICILLIUM* PARASITIC UPON *ASPERGILLUS NIGER*.

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(*Received December 1st, 1933.*)

THE mould whose behaviour on sugar solutions forms the subject of the present communication is a *Penicillium* originally isolated from an unsterilised cane sugar nutrient solution. Moulds believed to be of the same species have since been encountered contaminating *Aspergillus* cultures. The same or very similar species have also been found in nature accompanying *Aspergillus niger*. The association of the *Penicillium* with the latter mould is so intimate and its ultimate effect upon the *Aspergillus* so unfavourable that it may fairly be described as parasitic.

Thom [1930] states that members of the *P. rugulosum* series have frequently been found as parasites of other moulds in studies of the *Aspergilli*. Elsewhere Thom and Church [1926] refer a parasite of *A. niger* to the group *P. luteum-purpurogenum* close to *P. rugulosum*.

The present *Penicillium* bears conidia which resemble in size, shape and markings, and nearly in colour, those of *P. rugulosum* Thom. In some other respects, notably in its rapid and intense production of a diffusible red pigment in the mycelium, it resembles rather *P. purpurogenum* Stoll. On certain substrates, *e.g.* on malt agar, it shows a funiculose phase, forming ropes of red hyphae. On this medium there is often an early growth of pink or red pointed tufts of hyphae. Mature cultures on Czapek agar have herbage of a colour almost matched by "dull blackish green" of Ridgway, Plate XLI, m. 33, with here and there patches of yellow hyphae. The red pigment in the inferior surface and medium already referred to is very characteristic. Cultures have no marked odour. Colonies are not floccose. Sclerotia have not been observed. The penicillus is symmetrically biverticillate. The metulae, usually four in number, measure $11\mu \times 2{\cdot}5\mu$. The sterigmata $11\mu \times 2{\cdot}2\mu$. The conidial chains, which may be very long and densely packed, usually remain intact in fluid mounts. The conidia are elliptical ($3{\cdot}4\mu \times 2{\cdot}6\mu$), pigmented and have a roughened contour when ripe.

It is not proposed to attempt to identify the organism more closely than this, but to refer to it by the index letters under which it has been kept in culture, as *Penicillium* "*R.B.*"

In its action upon sugar solutions, *Penicillium* "*R.B.*" has been compared with type cultures of the other species named above. It is found to differ sharply from them by giving rise to an acid substance which is almost insoluble in water, but easily soluble in alcohol and other solvents specified later. Colourless crystals of this substance encrust the mycelium and glass in acid conditions. When chalk is present a soluble calcium compound forms in the solution. The explanation offered by Alsberg and Black [1913] for the distribution of mycophenolic acid according to the reaction of the medium probably applies here also.

As regards tolerance towards acidity *Penicillium* "*R.B.*" grows well on carbohydrate media over a large range of p_H and is tolerant of a high initial acidity in the medium. Thus, for example, it will grow well on sugar solutions in the presence of chalk and on similar sugar solutions having an initial p_H as low as 2·5. Nevertheless a certain acidity appears favourable for the germination of the conidia, and in the earlier experiments on chalk-containing media germination was quite erratic, frequently not taking place at all. On acidified media this trouble has never been experienced, and the difficulty with chalk was overcome by allowing germination to begin under acid conditions and then running the desired quantity of sterilised chalk suspension beneath the mould into the liquid. The conidia of many other moulds are known to germinate best in acid conditions [Webb, 1921]. The cultural device just mentioned may therefore have a more general interest.

The mould grows over a wide range of temperatures, with a growth-optimum probably at about 37°. It will grow at room temperature and 40°. Seed tubes kept over a year in cold store remained in perfectly good condition and gave rise to normal flask cultures.

The appearances of the cultures in the two cases where chalk is added after germination and where it is not added are very different. The biochemical changes and the fate of the sugar are also greatly affected as the following general description of the outstanding features at 28° in each case will show.

On chalk-containing sucrose medium, an opaque white mycelium forms comparatively rapidly. The inferior surface rapidly develops a blood-red colour which can be seen to diffuse into the solution. It begins first on those areas immediately over the chalk and can be hastened by disturbing the chalk from time to time. Sometimes the mould spores early, but will often remain free from spores to the end. Its surface then passes through various warm shades of colour—yellow, orange, rusty and red shades. Though the chalk is visibly disappearing there is little sign of any evolution of CO_2. The solution becomes more or less viscous. After some days white nodular crusts of calcium citrate separate out on the mycelium and the walls of the flask. Small quantities of calcium oxalate can also be demonstrated. After 10 days the solution may be run out and filtered from the calcium citrate and any remaining chalk. The filtrate gives a stronger calcium reaction than is accounted for by the dissolved calcium citrate. If it is acidified with HCl a cloud forms, often tardily, and on warming, coagulation and crystallisation take place. At this stage a characteristic aromatic rather pleasant smell is given off. When precautions are taken to decolorise the filtrate before these operations, these crude crystals are practically colourless.

On acidified sucrose medium the young mycelium is more frail and translucent and has a dangerous tendency to become "water-logged" and become submerged. Herrick and May [1928] record a similar observation with a variety of *P. purpurogenum* under certain conditions. *Penicillium* "*R.B.*" usually remains free from spores and shows more generous folding but produces no red colour. The solution is colourless, and in the early days becomes opalescent. It clears later, and small bunches of colourless crystals are found hanging from the mycelium or fallen to the bottom or adhering to the sides of the flask. From these may be obtained by purification and recrystallisation a substance which appears to be identical with "glauconic acid I" of Wijkman [1931].

The solution gives no detectable reaction for citric acid. The titratable acidity after the removal of the crystals is negligible. The crystals themselves show the same solubilities and behaviour as those derived from the calcium compound in the chalk cultures. If the inferior surface of the mycelium is touched with alkali, the bright red colour characteristic of the chalk-grown mycelium appears.

The substantial suppression under acid conditions of any accumulation of citric acid—an acid whose formation is known to be favoured by acid reactions in the case of other moulds [Currie, 1917; Butkewitsch, 1924]—and its accumulation when chalk is present is noteworthy. If oxalic acid is formed at all, it is only in traces. The presence of gluconic acid is not excluded.

Experimental.

Seed tubes for the inoculation of flask cultures have been grown on Czapek agar; malt-extract agar; and bread. Of the different temperatures used, 20°, 28° and 37°, 28° is probably the best for maintaining characteristics during subculture. 28° is also a satisfactory temperature for the incubation of flask cultures on sugar media.

(*a*) *Typical fermentation with* Penicillium "R.B." *on chalk-containing medium.*

Currie's solution, sucrose 150 g., NH_4NO_3 2·5 g., KH_2PO_4 1·0 g., $MgSO_4$, $7H_2O$ 0·25 g., HCl to p_H 3·4–3·5, water to 1000 ml. was used as a basis for culture solution. 250 ml. Erlenmeyer flasks containing 50 ml. of the solution were used. The initial p_H of the medium ensured sterility after 15–20 minutes' steaming at atmospheric pressure.

Five flasks inoculated from a bread culture of *Penicillium* "*R.B.*" grown at 28° were incubated at 30–31°. After 16 hours' germination conidia had formed a delicate surface veil. 10 ml. of a sterilised chalk suspension were then run into each flask by pipette and the cultures returned to the incubator. After 10 days the cultures were sterilised and the liquors filtered. Washings from the mould and calcium citrate and the residual chalk were added to the filtrate. Analysis of a small sample of the filtrate gave a total dissolved calcium of 0·823 g. Rather more than the equivalent of hydrochloric acid was then added, and the fluid became turbid. On boiling, a quantity of crude crystals weighing 1·8 g. was deposited. This is 4·8 % of the original sugar in solution.

Numerous flask experiments on this medium have been made at various temperatures. The water-soluble calcium compound is produced more freely at higher temperatures, 28°–37°, than at room temperature, and 28° was chosen. Other experiments on the same medium were made to find what differences resulted from adding the chalk suspension successively later and later after the germination of spores. Addition 12–18 hrs. after the beginning of incubation at 28° was found to give satisfactory types of culture. Other tests were made to detect differences due to agitating the chalk during incubation, without any decisive result—probably owing to the small depth of the solution. Among early fermentations many were designed to overcome the difficulty of irregular germination already mentioned.

The crude insoluble acid from most of these small tests was saved and bulked for purification later.

The solid calcium compounds were dissolved in excess of HCl, filtered and re-neutralised in the cold. A small precipitate, not wholly soluble on addition of acetic acid, formed. This was removed, and from it were obtained characteristic crystals of calcium oxalate. The calcium citrate in the solution was precipitated by boiling, filtered off and washed with boiling water. The dry weight of calcium citrate was 6·4 g. equivalent to 4·7 g. of citric acid; *i.e.* 12·5 % of the original sugar in solution.

Residual sugar was not estimated.

(*b*) *Typical fermentation with* Penicillium "R.B." *on acid medium.*

To examine the behaviour of *Penicillium* "*R.B.*" in absence of chalk a similar medium of the following composition was prepared and steamed for 20 mins. at atmospheric pressure: sucrose 150 g., NH_4NO_3 2·25 g., KH_2PO_4 0·875 g., $MgSO_4, 7H_2O$ 0·225 g., HCl 4·5 ml. of *N*/5, water to 1000 ml. Flasks, quantities of liquid and incubation temperature as before.

The mycelium had a slight peppering of olive-green spores interspersed with tufts of pale sulphur-yellow hyphae; it became only pale yellow on the under surface with traces of red at a few points on the rim in contact with the glass. After 11 days when the cultures were run out numerous small colourless crystals covered the under surface and the bottom of the flask.

Fermentation liquor and loose crystals were run out into a beaker, the bulk of those adhering to the mycelium were detached in running water and added to those in the beaker and all washed by decantation. They were filtered, washed with cold water and dried at 100°. The average weight crystallised from each flask was 0·125 g., 1·66 % of original sugar supplied. Melting-point of the crude crystals insoluble in water was between 193 and 197°. The average weight of dry mycelium from each flask was 0·28 g. The increase in the titratable acidity of the liquor was negligible. Qualitative tests for oxalic acid and citric acid were both negative. As before the sugar of the medium was not exhausted.

Many other small flask fermentations in absence of chalk have been carried out, varying the nutrients in solution, the temperature of incubation, concentration of sugar, p_H of solution and other factors. Many of these produced crude crystals. The yields reported are not large, but other experiments in progress indicate the possibility of considerably increasing the yield.

One solution which failed to yield any crystals was the Czapek-Dox solution. In this case, the cultures spored early and the fluid developed the red colour. The sugar in this solution is glucose, but that it was not the change from sucrose to glucose which caused the difference was proved by other tests.

Purification of the water-insoluble crystals. A quantity of crude crystals obtained as described above was dissolved in an excess of *N* NaOH. The solution was clarified with norite and filtered. The filtrate was acidified with strong HCl and an almost pure white product obtained, giving, after washing and drying at 100°, over 37 g. The melting-point of this material was not sharp, however, but ranged from 196 to 199°.

A part of this was submitted to recrystallisation from glacial acetic acid. This gave excellent crystals, the presumably purest specimens of which melted at 201–202°.

Properties. The substance is practically insoluble in cold water and only slightly soluble in hot water. It is insoluble in light petroleum. It dissolves in alcohol, acetone, ether, benzene, chloroform, pyridine and glacial acetic acid, from which it crystallises with ease. It dissolves in sodium carbonate solution with the evolution of CO_2, only gradually in dilute solutions of NaOH and may be reprecipitated with acid as described. It may also be recovered crystalline by adding water to a solution in alcohol and warming. It does not reduce Fehling's solution or ammoniacal silver nitrate. The bromine absorption test is negative, and it gives no coloration with $FeCl_3$. In concentrated H_2SO_4 it blackens on heating. It burns with a smoky flame.

Titration. The substance was dissolved in ethyl alcohol and titrated with *N*/10 NaOH in presence of phenolphthalein. In the cold there is no very definite

end-point; the alkali is only neutralised slowly. More soda is taken up on heating.

		Equivalent
Cold	0·0774 g. required 5·7 ml. *N*/10 NaOH	—
Hot	0·0774 g. required 8·55 ,, ,,	90·5
	0·1842 g. required 20·3 ,, ,,	90·7
	0·1591 g. required 18·2 ,, ,,	87·4

(Theoretical for $C_{18}H_{20}O_7$ titrating as a tetrabasic acid, 87·0.)

A search of the literature at the time failed to disclose any compound with the above properties produced by a mould. Before an opportunity occurred for a fuller chemical investigation of the new acid, Wijkman's work on the production of "glauconic acid I and II" by a *P. glaucum* appeared [Wijkman, 1931].

Her experiments were made on a sucrose solution containing very small quantities of mineral salts and without chalk present. In these circumstances she obtained insoluble acids crystallising out during the fermentation and from the crude mixture separated two pure substances, the properties of one of which, glauconic acid I, agree well with those of the acid produced by *Penicillium* "*R.B.*" Glauconic acid I is given the formula $C_{18}H_{20}O_7$, and has a melting-point of 202°.

A thorough chemical examination of the metabolic products of the *Penicillium* "*R.B.*" fermentation has still to be made, but the properties of the insoluble acid already described have since been compared with those described by Wijkman.

Mol. wt. 386, 380; micro-analysis (Schoeller) C, 62·07; H, 5·75 %; calculated for $C_{18}H_{20}O_7$; mol. wt. 348; C, 62·04; H, 5·79 %.

By acting on the acid dissolved in pyridine with acetic anhydride a crystalline derivative has been prepared whose melting-point closely approaches the value 175° given for the corresponding derivative of glauconic acid I. Micro-analysis C, 61·52; H, 5·64 %; calculated for mono-acetyl derivative C, 61·61; H, 5·68 %.

Heated above its melting-point, the original acid gives off a very fragrant vapour as the substance decomposes, as does glauconic acid I.

A substance corresponding to glauconic acid II has not been recovered, but the mother-liquors from the recrystallisation have not been investigated, so that the presence of this cannot be excluded.

The experiments already described were all made with sucrose. Fermentations were made upon 15 % solutions of glucose and fructose and in all cases crystals of the same appearance, giving the same qualitative tests as those obtained with sucrose, resulted. When chalk is present citric acid is also formed in quantities of the same order as with sucrose. Glycerol has also been tried, and the striking feature of the glycerol fermentation is that the solution instead of remaining colourless becomes a deep yellow.

Summary.

A *Penicillium* parasitic on *Aspergillus niger* is described. Its action upon sucrose solutions has been examined.

In the presence of chalk, considerable quantities of calcium citrate and small quantities of calcium oxalate are formed; also the soluble calcium salt of an acid insoluble in water, but soluble in alcohol.

In acid solutions the insoluble acid is formed encrusting the mould and separating on the vessel, but detectable quantities of citric and oxalic acids are not formed.

The insoluble acid also appears when sucrose is replaced in the medium by glucose or fructose. Citric acid is also formed from glucose and fructose when chalk is present.

The insoluble acid is probably to be identified with "glauconic acid I" of Wijkman, originally found in cultures of a *P. glaucum*.

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XXXI. PHENYL *ISO*CYANATE PROTEIN DERIVATIVES AND THEIR IMMUNOLOGICAL PROPERTIES.

III. THE AMINO-ACID DERIVATIVES AND SEROLOGICAL INHIBITION TESTS.

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(*Received January 1st, 1934.*)

In previous papers [Hopkins and Wormall, 1933, 1, 2] the chemical and immunological properties of phenylcarbamido-proteins have been recorded. It is to be presumed that in these compounds the $C_6H_5.NH.CO$-residue is attached to the free amino-groups of the lysine molecules. In view of the fact that serological inhibition tests have considerable value in the study of the constitution of chemically altered proteins, it was decided to make a more extensive study of the phenylcarbamido compounds. The phenyl- and *p*-bromophenylcarbamido-acids derived from many of the common amino-acids and similar compounds from α- and ε-amino-*n*-hexoic acid have therefore been prepared and have been used in precipitin and complement-fixation inhibition tests in the hope that more knowledge might be gained about the structure of proteins and the nature of the reaction between native proteins and phenyl *iso*cyanates. In particular it was hoped that these tests would give more information about the free basic groups of the intact protein molecule.

Preparation of the phenylcarbamido-acids.

These compounds were prepared by the action of phenyl *iso*cyanate (1·4 mol.) on the amino-acid (1 mol.) dissolved in water containing NaOH (1 mol.). Double quantities of NaOH were used for the preparation of the aspartic acid, cystine and tyrosine compounds and three times the normal quantity when lysine dihydrochloride was used. The amount of phenyl *iso*cyanate was doubled for the reactions with lysine, cystine and tyrosine, since the first two contain two amino-groups per molecule and tyrosine has a hydroxyl group which under certain circumstances might react with the *iso*cyanate.

The mixture of the *iso*cyanate and the solution of the sodium salt of the amino-acid was well stirred at room temperature (15–18°) for 1 hour, 0·5 *N* NaOH being added where necessary to maintain a slight alkalinity. The solution was then filtered, and the phenylcarbamido-acid was precipitated from the filtrate by the addition of dilute HCl, with cooling where necessary. Most of these compounds were recrystallised twice from water or dilute alcohol and dried *in vacuo* over $CaCl_2$. It was not found possible to recrystallise the lysine compound, and it was purified by reprecipitation. The yield of the recrystallised product was usually quite satisfactory. The melting-points recorded are all uncorrected.

The phenylcarbamido-acids used were all known compounds except those obtained from α- and ε-amino-*n*-hexoic acids which had M.P. 140–141° and 126–127° respectively.

Preparation of the p-*bromophenylcarbamido-acids.*

These compounds were prepared by the action of *p*-bromophenyl *iso*cyanate (1·4 mol.) dissolved in ether on the amino-acid (1 mol.) in slightly alkaline solution (1, 2 or 3 mol. of NaOH according to the amino-acid used, as in the case of the phenyl *iso*cyanate compounds).

The reaction mixture was cooled in ice and shaken for about 1 hour, more NaOH being added as necessary to keep the solution alkaline. The solution was then filtered and the *p*-bromophenylcarbamido-acid precipitated as before and recrystallised twice. It was not found possible to recrystallise the lysine and cystine compounds. Bromine estimations (Carius) and melting-point determinations were carried out on all the products. The results are tabulated below.

Properties of the p-*bromophenylcarbamido-acids.*

Amino-acid	M.P. of product	% Br Found	% Br Calc.	Crystalline form
Glycine	Decomposes 200–210° without melting	29·2	29·3 (for $C_9H_9O_3N_2Br$)	Flat prisms
Alanine	181–183° (decomp.)	27·7	27·9 (for $C_{10}H_{11}O_3N_2Br$)	Flat prisms
Leucine	162–164° ,,	24·3	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
α-Amino-*n*-hexoic acid	181–182° ,,	24·6	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
ε-Amino-*n*-hexoic acid	170–172° ,,	24·0	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Flat prisms
Lysine	176–177° ,,	28·5	29·5 (for $C_{20}H_{22}O_4N_4Br_2$)	Non-crystalline
Cystine	174–175° ,,	24·2	25·1 (for $C_{20}H_{20}O_6N_4S_2Br_2$)	Non-crystalline
Aspartic acid	175–176° ,,	24·5	24·2 (for $C_{11}H_{11}O_5N_2Br$)	Prismatic plates
Tyrosine	197–199° ,,	21·0	21·1 (for $C_{16}H_{15}O_4N_2Br$)	Flat prisms

The bromine values recorded in the above Table indicate that the normal reaction between *p*-bromophenyl *iso*cyanate and the amino-group of the amino-acid occurs with all the amino-acids tested. In the cases of cystine and lysine, the calculations are based on the introduction of two of these groups into each molecule of the amino-acid. The Br values obtained with these preparations were significantly lower than the calculated values, and it appears probable that these preparations consisted mainly of the disubstituted compounds with smaller amounts of the monosubstituted compound or compounds.

Inhibition tests.

Landsteiner [1920] first showed that some simple substances could specifically inhibit the formation of a precipitate when certain antisera were added to corresponding antigens. In these tests a neutral solution of the substance, which is to be tested for inhibitory power, is mixed with the antigen solution. To this

mixture is then added an antiserum which normally gives a marked precipitate with the antigen. If no precipitate is produced (*i.e.* complete inhibition), and if the substance does not inhibit any other type of precipitin reaction (*e.g.* that between horse-serum and an antiserum against horse-serum), specific union between antibody and inhibiting substance has occurred. This substance might therefore be said to have groupings similar to the antigenically prominent groupings of the antigen. The value of such tests will be discussed later in this paper.

Precipitin inhibition tests. Precipitin tests were carried out under the conditions described previously [Hopkins and Wormall, 1933, 1, 2]. In the inhibition tests, 0·05 ml. of the antigen solution (0·025 % phenylcarbamido-horse-serum-proteins or more dilute solutions of phenylcarbamido-gelatin) was added to 0·20 ml. of a carefully neutralised solution of the inhibitory substance (0·01, 0·005 or 0·0025 *M*). To this mixture was added 0·10 ml. of the antiserum (antiserum against phenylcarbamido-horse-serum-globulin) and the degree of precipitin formation noted immediately and after 5 mins., ½ hr. and 1 hr. These precipitin reactions were carried out at 37° except where the phenylcarbamido-gelatin compounds were used, these last-named compounds giving more pronounced precipitates at room temperature [Hopkins and Wormall, 1933, 2]. The results of the precipitin reactions are recorded as follows (in increasing degrees of precipitation): − (no reaction), f. tr. (faint trace), tr. (trace), ±, +, + ±, + +, *etc.*

Complement fixation inhibition tests. To a series of tubes each containing 0·5 ml. of diluted antigen solution, the range comprising 1:20, 1:60, 1:180 down to 1:1,180,980 of a 5 % antigen solution, were added 0·10 ml. of a neutral 0·01 *M* solution of the inhibiting substance (in 0·9 % NaCl solution), 0·10 ml. of 1:5 guinea-pig-serum and 0·05 ml. of the antiserum. After these mixtures had been allowed to stand for ¾ to 1 hr. at room temperature, 0·5 ml. of a 4 % suspension of sensitised ox red cells (in 0·9 % NaCl) was added to each tube. The tubes were immersed in a water-bath at 37° and the extent of haemolysis noted at intervals. In many experiments similar series of tests were made with larger or smaller amounts of guinea-pig complement, the results being essentially the same as those with the amount recorded above.

Antigens and antisera.

The preparation of the antigens and antisera used for this investigation has been described in previous papers [Hopkins and Wormall, 1933, 1, 2]. The antigen concentrations (1:20, 1:100, *etc.*) refer to dilutions of a 5 % solution.

Experimental.

The phenyl- and *p*-bromophenyl-carbamido-acids were tested for the power to inhibit the reaction between phenylcarbamido-proteins and corresponding antisera. The antisera used were those against phenylcarbamido-horse-serum-proteins, whereas the antigen was some other phenylcarbamido-protein, either phenylcarbamido-chicken-serum-proteins or phenylcarbamido-gelatin. The last-named antigen proved of special value for this purpose, since with the antisera mentioned it gives marked precipitates in very high dilutions, and in these inhibition tests it is of advantage to reduce the amount of antigen to the minimum necessary to give a good precipitin reaction. In this way it is possible to reduce the amount of antigen competing with the inhibiting substance, and any

inhibitory action by the latter is usually more definite with the lower concentration of antigen.

These inhibition tests have been made with several concentrations of the antigen and antisera and with different amounts of the substances tested for inhibitory power. The last-named were usually used in 0·01 *M* solution in 0·9 % NaCl, this being about the maximum solubility for some of the substances. The results of a typical experiment are given in Table I, this Table giving readings with two concentrations of antigen and two concentrations of the inhibiting substances.

Table I. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins.
Immune-serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).
Temperature: 37°.

	Antigen solution			
	1 : 20	1 : 20	1 : 100	
	Solution of inhibiting substance			
			0·01 *M*	
Inhibiting substance	0·01 *M*	0·005 *M*		
NaCl	+ ±	+ ±	+ ±	+ ±
Glycine	+ ±	+ ±	+ ±	+ ±
Alanine	+ ±	+ ±	+ ±	+ ±
α-Amino-*n*-hexoic acid	+ ±	+ ±	+ ±	+ ±
Lysine	+ ±	+ ±	+ ±	+ ±
Phenylcarbamido-acetic acid	+	+	tr.	±
α-Phenylcarbamidopropionic acid	+	+	tr.	±
α-Phenylcarbamido-*n*-hexoic acid	+	+	tr.	±
αε-Diphenylcarbamido-*n*-hexoic acid	±	±	−	f.tr.
αε-Diphenylcarbamido-*n*-hexoic acid*	±	±	−	f.tr.
p-Bromophenylcarbamido-acetic acid	+ ±	+ ±	tr.	±
Readings after:	30 mins.	30 mins.	5 mins.	30 mins.

* Tests with one-half of the corresponding 0·01 or 0·005 *M* solutions.

From this table it will be seen that the phenylcarbamido-acids have a marked inhibitory influence on the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera, and that the most pronounced effect is exhibited by the lysine derivative. Preliminary experiments of this nature were used in support of the view that the reaction between phenyl *iso*cyanate and proteins involves the free amino-groups of the lysine molecules of the protein [Hopkins and Wormall, 1933, 1]. Since lysine has two NH_2-groups it seems possible that the corresponding phenylcarbamido-acid might be most efficient as an inhibitory agent because it contains two phenylcarbamido-groupings per molecule, but this explanation was soon excluded, for the lysine compound always gives much better inhibition than does twice the equivalent amount of the other α-phenylcarbamido-acids. In the experiments described in this paper, tests with one-half the equivalent amounts of the lysine compounds were always made, the results being identical with those obtained with the higher concentrations of these compounds.

The results given in Table I demonstrate the advantage of using as little antigen as possible for these inhibition tests, although it is necessary, of course, to obtain a good precipitate in the control tests, and they indicate also the added information which is gained by taking readings at various intervals of time. The results with *p*-bromophenylcarbamido-acetic acid show that this compound has some inhibitory action on the phenylcarbamido-precipitin reaction, but that the effect is less marked than that produced by phenylcarbamido-acetic acid.

One of the main objects of the work described in this paper was to obtain as much information as possible, using immunological methods, about the free basic groups of the protein molecule. For this purpose α- and ε-amino-*n*-hexoic acids and their phenyl- and *p*-bromophenyl-carbamido-derivatives were prepared and these compounds and the corresponding lysine compounds compared

Table II. *Precipitin inhibition tests.*

Immune serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).
Temperature: 16°. (Readings after 30 mins.).

Inhibiting substance		Antigen: Phenylcarbamido-gelatin (1 : 500)		Antigen: *p*-Bromophenylcarbamido-gelatin (1 : 500)
NaCl		+ ±	+ ±	+
Alanine (lysine, or α- or ε-amino-*n*-hexoic acid)		+ ±	+ ±	+
Phenylcarbamido-acid corresponding to	alanine	tr.	+	tr.
	lysine	–	–	–
	α-amino-*n*-hexoic acid	tr.	+	tr.
	ε-amino-*n*-hexoic acid	–	–	–
Concentration of inhibiting substance:		0·01 *M*	0·0025 *M*	0·01 *M*

Note. None of the amino-acids used in this investigation, or the corresponding phenylcarbamido-acids, had any inhibitory influence in these concentrations (0·01 *M* and more dilute solutions) on the precipitin reaction between horse-serum-globulin and an antiserum to horse-serum-globulin.

Table III. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins (1 : 100) and phenylcarbamido-gelatin (1 : 500).
Inhibiting substance: Neutralised 0·01 *M* solutions (in 0·9 % NaCl).
Antiserum: Antiphenylcarbamido-horse-serum-globulin (No. 94).
Temperature: 37° for the chicken-serum compound and 16° with phenylcarbamido-gelatin as antigen.

Inhibiting substance		Antigen: Phenylcarbamido-chicken-serum-proteins		Antigen: Phenylcarbamido-gelatin	
NaCl		+ +	+ +	+	+
Cystine (tyrosine, leucine, aspartic acid, α- and ε-amino-*n*-hexoic acids and lysine)		+ +	+ +	+	+
Phenylcarbamido-acid corresponding to	cystine	–	–	–	–
	tyrosine	tr.	tr.	–	–
	leucine	tr.	tr.	–	–
	aspartic acid	+	+	±	±
	α-amino-*n*-hexoic acid	–	–	–	–
	ε-amino-*n*-hexoic acid	–	–	–	–
	lysine	–	–	–	–
	lysine*	–	–	–	–
p-Bromophenylcarbamido-acid corresponding to	cystine	tr.	±	–	f.tr.
	tyrosine	tr.	±	f.tr.	f.tr.
	leucine	±	±	–	–
	aspartic acid	±	±	f.tr.	f.tr.
	α-amino-*n*-hexoic acid	±	±	–	–
	ε-amino-*n*-hexoic acid	–	–	–	–
Readings after:		½ hr.	1 hr.	½ hr.	1 hr.

* *Cf.* Note to Table I.

Note. Smaller amounts of the phenylcarbamido-acids derived from cystine, tyrosine, leucine, aspartic acid and α-amino-*n*-hexoic acid gave slight inhibition only, whereas with the compounds from lysine and ε-amino-*n*-hexoic acid the inhibition was still complete.

as inhibitors of the phenylcarbamido-precipitin reaction. From the results of a typical experiment of this nature (*cf.* Table II), it appears that of these two monoamino-*n*-hexoic acids the ϵ-compound is the one which most resembles the lysine compound in its inhibitory power.

The results recorded in Table III are similar to those in Tables I and II. They show that all the phenylcarbamido-acids tested have a marked inhibitory effect on the precipitin reaction between phenylcarbamido-proteins and corresponding antisera, whereas the amino-acids themselves are inactive. The phenylcarbamido-acid derived from aspartic acid gave moderate inhibition only when compared with equivalent amounts of the corresponding compounds from glycine, cystine, *etc.*, but this is probably due to the fact that the preparation obtained by the action of phenyl *iso*cyanate on aspartic acid is not very pure, and the recrystallised material represents a mixture of the free acid and the hydantoin [Paal and Zitelmann, 1903]. Thus all the phenylcarbamido-acids with the exception of the aspartic acid derivative gave complete or almost complete inhibition in the concentration used in the experiment recorded in Table III. With more dilute solutions of the inhibiting substances, the lysine and ϵ-amino-*n*-hexoic acid derivatives showed more marked inhibitory power than did those of the other amino-acids, as in the experiments recorded in Table II.

Table III also gives the results of tests with the *p*-bromophenylcarbamido-acids. These compounds, although not quite as effective as the corresponding phenyl compounds, possess a marked inhibitory capacity. The lysine derivative could not be used in these tests on account of its very slight solubility in water, but of the other compounds tested, the ϵ-amino-*n*-hexoic acid derivative was most effective. The introduction of bromine into the benzene ring of the phenylcarbamido-acids thus reduces somewhat but does not destroy the capacity of these substances to react with the antibodies to phenylcarbamido-proteins. The phenylcarbamido-acids are able similarly to inhibit the precipitin reaction between *p*-bromophenylcarbamido-gelatin (or the corresponding horse-serum compound) and antibodies to phenylcarbamido-proteins.

In Table IV a typical result is given showing the influence of some of the phenylcarbamido-acids on complement fixation tests, the other derivatives not

Table IV. *Complement fixation tests* (*inhibition tests*).

	Inhibiting substance																	
											Phenylcarbamido-acid corresponding to							
	NaCl		Glycine		α-Amino-*n*-hexoic acid		ϵ-Amino-*n*-hexoic acid		Lysine		Glycine		α-Amino-*n*-hexoic acid		ϵ-Amino-*n*-hexoic acid		Lysine	
Antigen dilution																		
1 : 20	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 60	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 180	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 540	–	2	2	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 1620	–	–	–	–	–	–	–	1	–	–	3	4	4	4	4	4	4	4
1 : 4,860	–	–	–	–	–	–	–	–	–	–	2	3	3	4	4	4	4	4
1 : 14,580	–	–	–	–	–	–	–	–	–	–	–	3	–	3	3	4	4	4
1 : 43,740	–	–	–	–	–	–	–	–	–	–	3	4	1	3	4	4	4	4
1 : 131,220	–	1	–	–	–	–	–	–	–	–	3	4	3	4	4	4	4	4
1 : 393,660	2	4	–	–	–	–	–	–	–	–	4	4	3	4	4	4	4	4
1 : 1,180,980	4	4	–	1	–	1	–	1	–	1	4	4	4	4	4	4	4	4
Control (NaCl)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Reading after (hr.)	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½

In the above table, 4 represents complete haemolysis, – no haemolysis, and 3, 2 and 1 intermediate degrees of haemolysis.
Phenylcarbamido-gelatin was the antigen used for these tests and the antiserum was one against phenylcarbamido-horse-serum-globulin.

quoted in this Table giving results similar to those with that prepared from glycine. Thus, these compounds are capable of effecting marked inhibition of the fixation of complement by mixtures of phenylcarbamido-gelatin and antisera to phenylcarbamido-proteins. This other type of inhibition test, involving inhibition of complement fixation, appears to give even more conclusive results than do the corresponding precipitin inhibition tests, and they confirm the finding that the phenylcarbamido-acids from lysine and ϵ-amino-*n*-hexoic acid are the most active of the amino-acid derivatives tested. In these complement fixation tests, as with the corresponding precipitin inhibition tests, experiments were made repeatedly to show that the inhibitory action of the phenylcarbamido-acids is specific, and that these compounds do not inhibit the interaction (in complement fixation and precipitin tests) between other antigens and their antibodies.

Discussion.

Specific inhibition of the precipitin reaction by simple substances was first described by Landsteiner [1920], and since then this reaction has been used with much success by Landsteiner and van der Scheer [1928; 1931; 1932, 1, 2] and by other authors. Specific inhibition is obtained when the added substance contains a grouping identical with, or related to, that which is antigenically active in the antigen. Thus, *p*-amino- and *p*-bromo-benzoic acids inhibit the precipitin reaction between *p*-aminobenzoic acid-azo-protein and its antibody, but much less inhibition is exerted by *o*-amino- or *o*-bromo-benzoic acid [Landsteiner, 1920]. In a similar manner, the reaction between azo-proteins formed by the coupling of a protein with diazotised aminobenzoyl-dipeptides (*e.g.* *p*-aminobenzoylglycylglycine) and antibodies to these compound proteins is specifically inhibited by the corresponding *p*-amino- or *p*-nitro-benzoyl-dipeptide [Landsteiner and van der Scheer, 1932, 1]. In many instances therefore it has been shown that simple substances can unite with antibodies and prevent, partially or completely, the reaction with the homologous antigen, and such tests are very helpful in studies on the interaction between antigen and antibody and in investigations concerned with immunological specificity. Such tests may also be used for more strictly chemical purposes. Thus, the inhibition of the reaction between iodinated proteins and their antisera by 3 : 5-dihalogenated tyrosine furnished very strong evidence in support of the view that the method of iodination used resulted mainly in the introduction of two iodine atoms into the 3 : 5-positions of each tyrosine grouping [Wormall, 1930].

From the investigations referred to above it will be seen that these inhibition tests can reveal much about the nature of groupings in chemically altered proteins, or proteins to which new groupings have been attached, and it appears highly probable that they will help considerably in attempts to learn more about the intact protein molecule. The tests do not involve any drastic treatment of the material to be tested, and they also allow the recognition of substances which cannot readily be isolated in the pure condition. Thus "inert" substances do not interfere with the inhibitory action of other substances, *e.g.* the specific inhibition of the phenylcarbamido-precipitin reaction by phenylcarbamido-acids, described in this paper, is not affected by the presence of unchanged amino-acids, *etc.* The tests can readily be used, therefore, to detect the presence of certain substances in a fairly complex mixture. They can also be used, in addition to the ordinary precipitin tests, in studies on spatial configuration [Landsteiner and van der Scheer, 1932, 3].

The work described in this paper has been concerned mainly with the free amino-groups of the protein molecule. Phenyl *iso*cyanate readily reacts with

peptones as was shown by Raper [1907], who first described these compounds, and a similar reaction occurs with native proteins [Hopkins and Wormall, 1933, 1, 2]. The reaction with native proteins will obviously be more restricted, since the number of free amino-groups in the molecule of a protein of this type is usually rather small, and with a protein like zein, which is lacking in these basic groups, no reaction occurs. In the phenylcarbamido-proteins therefore the new groups appear to be attached to the free amino-groups only, and evidence of a chemical and immunological nature was put forward in support of the view that the main new grouping in these protein compounds is attached to the ϵ-amino-group of lysine [Hopkins and Wormall, 1933, 1]. Lysine however is a diamino-acid ($\alpha\epsilon$-diamino-*n*-hexoic acid), and it would be very helpful if we could determine whether the new groupings are attached to the α- or the ϵ- or both the α- and ϵ- amino-groups of the lysine molecules: in other words, which amino-groups are free in the intact protein. The chemical evidence available suggests that the free grouping is the ϵ-amino-group [*cf.* Van Slyke and Birchard, 1913; Edlbacher, 1921; Goldschmidt and Kinsky, 1929; Kapfhammer, 1930; Abderhalden and Schweitzer, 1931].

This view about the free amino-groups of the intact protein molecule receives very strong support from the immunological evidence presented in this paper. Inhibition tests (precipitin and complement fixation) show that $\alpha\epsilon$-diphenylcarbamido-*n*-hexoic acid combines very readily with antibodies to phenylcarbamido-proteins, and that similar phenylcarbamido-acids derived from the common amino-acids (glycine, cystine, tyrosine, *etc.*), although they are quite active in this respect, do not show by any means the same degree of activity as the lysine derivative. Furthermore, a comparison of the phenylcarbamido-acids from α- and ϵ-amino-*n*-hexoic acids has demonstrated that the latter behaves like the lysine derivative and the former like those of the other amino-acids. Thus the configuration of ϵ-phenylcarbamido-*n*-hexoic acid shows most resemblance to that configuration in phenylcarbamido-proteins which reacts with phenylcarbamido-antibodies, which leads one to the conclusion that a large number of the ϵ-amino-groups of the lysine components of the protein are free. The marked difference between the inhibitory powers of the lysine and the ϵ-amino-*n*-hexoic acid derivatives on the one hand, and derivatives of the other amino-acids on the other, suggests that the majority, if not all, of the ϵ-amino-groups of the lysine are free, but this conclusion must await further experimental evidence. Although this particular problem might not be capable of final solution by serological tests similar to those used in this investigation, sufficient has been said here to indicate the value of such tests in studies on the chemistry of proteins.

Summary.

1. Phenyl and *p*-bromophenyl *iso*cyanate derivatives of several amino-acids have been prepared, including those of α- and ϵ-amino-*n*-hexoic acids.

2. Inhibition by these phenylcarbamido-acids of the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera has been studied. Similar inhibition of the complement fixation reactions has been obtained.

3. The phenylcarbamido-acids from lysine and ϵ-amino-*n*-hexoic acid give complete inhibition, whereas equivalent amounts of the derivatives of α-amino-*n*-hexoic acid, leucine, alanine, tyrosine, *etc.*, give moderate inhibition only.

4. These serological inhibition tests offer, therefore, strong support for the view that the free amino-groups of the intact protein molecule are the ε-amino-groups of the lysine molecules.

One of the authors (A. W.) is indebted to the Medical Research Council for a part-time personal grant and for a grant which has, in part, defrayed the expenses of this research.

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XXXII. STUDIES ON XANTHINE OXIDASE.
XI. XANTHINE OXIDASE AND LACTOFLAVINE.

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(*Received January 2nd, 1934.*)

Until recently the view was held by many that molecular oxygen cannot react directly with the reducing systems of the cell but requires the intermediation of catalytic iron. Experiments on oxidase systems extracted from cells seemed to support this view. The succinic oxidase of muscle, for instance, as shown by Thunberg [1917] and others, will reduce methylene blue or oxygen in presence of succinate, but if a small amount of HCN is added all power of reducing oxygen is lost, while methylene blue is still reduced with the same velocity as before. This was interpreted as being due to the inactivation of the iron compound acting as an intermediary between the activated substrate and molecular oxygen.

The xanthine oxidase of milk and liver was however shown by Dixon and Thurlow [1925] to be unaffected by HCN, both with respect to methylene blue reduction and oxygen uptake. This system was therefore assumed to act in accordance with Wieland's theory of oxidation, in which molecular oxygen acts as a direct acceptor of the hydrogen of the substrate molecule.

The work of Warburg and Christian [1932] has shown the existence of another type of compound in cells which functions as an intermediary between the reducing systems and oxygen. This is a yellow pigment which does not contain iron or any other metal. It is rapidly reducible by the hexosemonophosphate dehydrogenase system isolated from several kinds of cells and is then reoxidised by molecular oxygen with the formation of H_2O_2; it can function catalytically even in the presence of HCN.

The recent researches of Kuhn *et al.* [1933] and Stern [1933] have shown that yellow pigments of this type, now known as flavines, occur in both milk and liver, and the possibility therefore arose that in the xanthine oxidase system prepared from these sources the oxygen reacted directly with the leuco-form of the yellow pigment and not directly with the substrate. The formation of H_2O_2 observed by Thurlow [1925] in this system might be accounted for by this reaction. If such were the case the xanthine oxidase could no longer be regarded as supporting the Wieland view.

It therefore became important to ascertain whether the uptake of oxygen by the xanthine oxidase system was dependent upon the presence of such yellow pigments.

This question could be settled experimentally in the following ways. (1) By complete removal of yellow pigment from xanthine oxidase preparations. This should abolish the power of taking up oxygen. (2) By addition of yellow pigment to pigment-free preparations, which should increase the rate of oxygen uptake. (3) By determining the ratio, rate of methylene blue reduction/rate of oxygen uptake, in a number of xanthine oxidase preparations prepared in different ways,

and comparing its value with the pigment content of the preparations. The smaller the pigment content, the larger should be the ratio. (4) By determining the power of the oxidase to reduce the yellow pigment.

Experimental methods.

Preparations of xanthine oxidase.

A. *Caseinogen preparation* [Dixon and Thurlow, 1924, 1].

The enzyme is precipitated together with the caseinogen and fat by half-saturation of the milk with ammonium sulphate. The fat is removed from the dried precipitate by thorough extraction with ether.

B. *Cream preparation* [Toyama, 1933].

Cream is separated from milk. The fat is dissolved away with ether, and the protein residue is dried in the desiccator.

C 1. *Whey preparation* [Dixon and Kodama, 1926].

Fresh milk is clotted by rennin, the enzyme precipitated from the whey by half-saturation with ammonium sulphate and the precipitate extracted with ether and dried.

C 2. *Charcoal-treated whey preparation* [Dixon and Kodama, 1926].

A solution of the C 1 preparation is treated twice with Merck's medicinal charcoal, and the enzyme is precipitated from the clear filtrate with ammonium sulphate. Most of the protein and all the yellow pigment are removed by this procedure.

C 3. *Kaolin-treated whey preparation* [Dixon and Kodama, 1926].

The enzyme is adsorbed from a solution of the C 1 preparation at p_H 5 with kaolin and subsequently eluted from the kaolin with 1 % Na_2CO_3. It is then precipitated as before with ammonium sulphate. This procedure also removes most of the protein and all the yellow pigment.

C 4. *Fuller's earth-treated whey preparation.*

Fuller's earth is known to adsorb the yellow pigment strongly and is used for its concentration from milk. A solution of the C 1 preparation is treated twice with fuller's earth (Boots). The filtrate contains practically all the enzyme, no yellow pigment and about half the original protein. The enzyme is precipitated as before with ammonium sulphate.

D. *Sodium chloride preparation* (Dixon and Lemberg, unpublished).

The caseinogen and fat are precipitated from milk with sodium chloride, leaving the enzyme in solution, from which it is precipitated with ammonium sulphate. This gives a preparation of high activity containing a small amount of the yellow pigment. The complete details of the method will be published shortly.

Preparation of lactoflavine.

Ten litres of milk were acidified to p_H 4·6 with glacial acetic acid, and the caseinogen was filtered off. The filtrate was mixed with 500 g. of fuller's earth, which was filtered off after 1 hour and washed three times with 1 litre of distilled water and twice with 1 litre of ethyl alcohol. The lactoflavine was then eluted, following the procedure of Kuhn *et al.*, with a mixture of pyridine, acetic acid

and methyl alcohol. After centrifuging off the fuller's earth, the yellow solution was concentrated *in vacuo* to about 25 ml. and mixed with an equal volume of distilled water. The solution was then thoroughly extracted with ether, the yellow pigment remaining in the aqueous solution. The small precipitate which formed was filtered off. An equal volume of methyl alcohol was then added to the opalescent solution, causing the precipitation of a small amount of colloidal fuller's earth, which was filtered off. The yellow solution at this stage was perfectly clear and fluoresced strongly. It was then concentrated *in vacuo* to about 15 ml. and mixed with 10 volumes of acetone. The whole solution set to a gel, which could be easily broken up and filtered. The clear filtrate was again concentrated *in vacuo* to 15 ml., and phosphate solution (p_H about 7·4) was added until no more precipitate was formed. The precipitate was filtered off and the filtrate used in subsequent experiments. By colorimetric comparison with a lactoflavine solution of known concentration kindly given to us by Dr György it was found that the solution contained 50γ of lactoflavine per ml. The yield from 10 litres of milk was therefore 1 mg. of lactoflavine.

Detection of lactoflavine in xanthine oxidase preparations.

The dried enzyme preparations were extracted with small volumes of either methyl alcohol or pyridine, and the extracts were examined for greenish fluorescence in a strong beam of ultra-violet radiation, obtained from a mercury arc lamp with a nickel oxide filter. This test is exceedingly delicate, particularly when methyl alcohol is used, and 0·05γ of lactoflavine per g. of enzyme preparation can be detected with ease. In some cases aqueous solutions of the preparations were also tested for fluorescence.

Measurement of rate of reduction of methylene blue.

For the Thunberg technique to serve as a quantitative measure of the rate of reduction of methylene blue, the following conditions must be fulfilled. (1) The solutions must be completely freed from all traces of dissolved oxygen. (2) The substrate must not be mixed with the enzyme solution prior to evacuation of the tubes. (3) Temperature equilibrium must be attained by the tubes before the experiment begins.

The procedure adopted was as follows. Thunberg tubes of the Keilin type were used, and the hypoxanthine was placed in the hollow stopper. Four tubes were used for each determination. These were evacuated simultaneously with a water-pump, filled with oxygen-free nitrogen from a reservoir and allowed to come into equilibrium with the gas before being again exhausted. This process was twice repeated. The tubes were placed in a thermostat at 37°. The stoppers were worked in and the side-tubes filled with water in order to prevent air leakage. After three minutes the tubes were tilted simultaneously so as to mix the enzyme and substrate. By this procedure duplicates were found to agree to within 5 % in all cases.

The xanthine oxidase presents special difficulties in the determination of the rate of reduction of methylene blue, owing to the peculiar effects which are produced by changes of substrate concentration. Dixon and Thurlow [1924, 2] showed that over a certain range of hypoxanthine concentration the rate is maximum and independent of the concentration, but that with higher concentrations of substrate an inhibition ensues. A single determination, therefore, is insufficient to determine the true activity of the enzyme, and it is necessary in every case to work with two different concentrations of hypoxanthine, in order to ensure that the maximum rate is being measured.

The strength of the methylene blue solution used was determined by colorimetric comparison with a standard solution of a twice recrystallised specimen of pure medicinal methylene blue of known nitrogen and moisture content.

Measurement of rate of uptake of oxygen.

The measurements were carried out in the Barcroft apparatus at 37° by the usual technique [Dixon, 1934]. The hypoxanthine was placed in "Keilin tubes" and mixed with the enzyme only after temperature equilibrium was attained. Air, and not oxygen, was used in the flasks, as control experiments showed that the rate was independent of the partial pressure of oxygen within wide limits. Any traces of CO_2 were absorbed by KOH-papers in the usual way. Here again experiments with different hypoxanthine concentrations must be carried out, as the effects of substrate concentration are similar to those observed with methylene blue.

Procedure.

Suitable amounts of dried enzyme powder were dissolved in $M/5$ phosphate buffer (p_H 7·77) and diluted so as to give convenient rates of reduction. Barcroft and Thunberg experiments were carried out simultaneously, and each experiment was always done in duplicate.

Experimental results.

The yellow pigment can be removed from the oxidase preparations by adsorption with charcoal, kaolin or fuller's earth, and also by dialysis. For instance, one adsorption by any of these adsorbents is sufficient to remove the pigment from the C 1 preparation so completely that no trace can be detected by the fluorescence test when the oxidase is subsequently precipitated and 5 g. of the dried precipitate extracted with methyl alcohol. Nevertheless, after three successive treatments with charcoal the enzyme is found to have lost none of its power of taking up oxygen, and treatment with kaolin or fuller's earth likewise leaves the rate of oxygen uptake unaffected. It is therefore clear that the oxygen uptake does not depend upon the presence of lactoflavine.

In order to determine whether the pigment influences the rate of oxygen uptake in any way, the ratio, rate of methylene blue reduction/rate of oxygen uptake, was determined for the various preparations of the oxidase and compared with their content of yellow pigment. The results are summarised in Table I.

Table I.

Preparation	Degree of concentration of oxidase	Presence of lactoflavine	M.B. reduced in 20 mins. (mm.^{3} O_2)	O_2 absorbed in 20 mins. (mm.^{3})	Ratio
Milk	1	+ + + +	76	125	0·61
A	30	+ + +	19	34	0·56
B	400	–	36	63	0·57
C 1	500	+?	230	328	0·70
C 2	2000	–	98	174	0·56
C 3	2000	–	111	181	0·61
C 4	1000	–	87	119	0·73
D	2000	+	195	322	0·61

The figures given in the second column of the table represent very approximately the degree of concentration given by the different methods of preparation, *i.e.* they give the number of units of enzyme in 1 g. of the solid compared with 1 g. of the original milk. A *minus* sign in the third column means that no trace

of lactoflavine could be detected in 5 g. of the solid by the procedure given above. The amounts of methylene blue reduced have been expressed in mm.^{3} of oxygen, on the assumption that one molecule of methylene blue is equivalent to one atom of oxygen, in order to be able to compare the two rates. The figures given in the fourth column represent the amounts which 2·5 ml. of enzyme solution would reduce in 20 minutes, calculated from the observed rate of reduction of 1 ml. of methylene blue (M/1500). The amounts of oxygen given in the fifth column are obtained from the initial linear portions of the oxygen uptake curves and represent the amounts absorbed by 2·5 ml. of the enzyme solutions in 20 minutes at the same temperature. Since the solutions of the various preparations of enzyme were arbitrarily diluted to different extents, so as to give convenient rates, the figures given in columns 4 and 5 give no index of the relative activities of the different preparations in the solid state and cannot be compared with one another.

It will be seen that the ratio of the two velocities remains remarkably constant, in spite of wide variations in the content of lactoflavine.

The addition of concentrated lactoflavine solution to preparations which had been freed from yellow pigment by dialysis or adsorption produced no increase in the rate of oxygen uptake, as shown in Tables II and III.

Table II.

Barcroft manometer	1	2	3	4
Dialysed oxidase (preparation A) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·3	0·1	0·1
O_2 uptake in 20 mins. (mm.^{3})	38·0	37·4	35·7	36·6

Table III.

Barcroft manometer	1	2	3	4
Oxidase (preparation C 2) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·2	0·1	0·1
O_2 uptake in 10 mins. (mm.^{3})	81·7	74·5	79·6	79·8

Table IV.

Thunberg tube	1	2	3	4
Oxidase (preparation A) (ml.)	1·0	1·0	1·0	1·0
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	1·0	1·0	—	—
Methylene blue (0·00034 M) (ml.)	—	—	1·0	1·0
Reduction time	35 mins.	36 mins.	70 secs.	70 secs.

The power of the xanthine oxidase to reduce lactoflavine anaerobically was then tested by comparing the times of reduction of 60γ added lactoflavine and methylene blue in Thunberg tubes. The times given in Table IV are those for complete reduction as shown by complete disappearance of colour. It can be easily calculated that lactoflavine is reduced with a velocity of only 1/64th of that of methylene blue reduction.

Discussion.

The results given above show clearly that lactoflavine plays no part in the oxygen uptake by the xanthine oxidase. The rate of uptake is unaffected alike by complete removal and by addition of the pigment, and the ratio of the reaction rates with methylene blue and oxygen does not change with the pigment content.

An incredibly high catalytic activity would have to be assumed if traces of the yellow pigment, too small to be detected, are to account for the observed oxygen uptake. In preparation C 3, for example, certainly less than 0·05γ per g. was present. 10 mg. of the preparation, containing less than 0·0005γ of lactoflavine, were used in the experiment given in Table I, and took up 181 mm.^{3} in 20 minutes, or 9 mm.^{3} per minute. As 0·0005γ of lactoflavine is equivalent to 0·000015 mm.^{3} of oxygen, the lactoflavine would have to be reduced and oxidised 600,000 times per minute in order to account for the oxygen uptake, whereas Warburg and Christian found that the corresponding catalytic flavines of bottom yeast and lactic acid bacteria were reduced and oxidised only 24 and 30 times per minute respectively.

The observed rate of reduction of lactoflavine by xanthine oxidase also shows that it cannot account for the oxygen uptake. Table IV shows that an amount of lactoflavine corresponding to 1·8 mm.^{3} of oxygen was reduced in 35 minutes. In the same period this solution of oxidase would have taken up about 210 mm.^{3} of oxygen, so that it is clear that even this relatively large amount of pigment could only account for less than 1 % of the oxygen uptake.

Since Dixon and Thurlow showed that the xanthine oxidase differed from the majority of the oxidases, in that its ability to react with molecular oxygen was not inhibited by HCN, it has been regarded, in common with the other cyanide-stable respiration systems, as a case in which the oxygen reacted directly with the activated substrate molecules in accordance with Wieland's theory. Warburg and Christian [1933] have, however, shown that at any rate in some of these cases, which the haematin catalysis theory could not explain, the oxygen uptake could be accounted for by the action of catalytic flavines (which are resistant to cyanide), the oxygen reacting with these and not directly with the organic substrates.

Warburg and Christian assert that, just as the Wieland theory is wrong in all those cases in which the respiration is inhibited by HCN (for the oxygen then reacts, not with the substrate, but with the reduced form of a haematin), it is no less wrong in those (rarer) cases in which the respiration is not inhibited by HCN (for the oxygen then reacts, again not with the substrate, but with the leuco-form of the flavine); and they state that the idea of a direct reaction of molecular oxygen with the substrate must now be abandoned.

In the xanthine oxidase, however, we have an oxidase system whose oxygen uptake is due neither to haematin nor to flavine, but apparently to a direct reaction of oxygen with the activated substrate molecules.

Summary.

The ability of the xanthine oxidase of milk to react with molecular oxygen is not dependent upon the presence of lactoflavine. In this system the molecular oxygen probably reacts directly with the activated substrate molecules.

One of us (D. E. G.) wishes to thank the Trustees of the George Henry Lewes fund for a research grant.

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XXXIII. STUDIES IN TISSUE METABOLISM.
III. THE EFFECT OF OXIDISED 1:2:5:6-DIBENZANTHRACENE.

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(*Received January 2nd, 1934.*)

By means of the Thunberg technique oxidation products of carcinogenic hydrocarbons were shown to inhibit lactic and succinic dehydrogenases of yeast and muscle extracts in the presence of methylene blue as hydrogen acceptor [Boyland, 1933]. Qualitative examination of the effect on the oxidation of *p*-phenylenediamine by washed tissue indicated that these hydrocarbons did not inhibit indophenol oxidase. The enzymes used in the previous experiments had been used in the form of extracts. The object of the present work was the investigation of the effect of a water-soluble oxidation product of the carcinogenic hydrocarbon, 1:2:5:6-dibenzanthracene [Cook *et al.*, 1932] on the respiration and anaerobic glycolysis of surviving animal tissues (normal organs and tumours of rat and mouse) using the Warburg technique. The tissues were suspended in Ringer-phosphate or $M/15$ phosphate solutions (p_H 7·3), either in slices less than 0·4 mm. thick or finely minced, in the vessels of a Warburg apparatus; the amounts of oxygen absorbed and the anaerobic glycolysis were measured in the presence of varying concentrations of the oxidation product.

Experimental.

1:2:5:6-Dibenzanthracene (0·1 % in benzene) was oxidised by passing a stream of oxygen through the solution while exposed to ultra-violet light for 48 hours. A light brown precipitate was formed, and the solution became darker. The precipitate and solution were extracted with aqueous $2N$ NaOH, and the extract was precipitated with cold hydrochloric acid. The brown precipitate was washed with water and dried; weighed quantities were dissolved either (1) in phosphate buffer solution ($M/5$; final p_H 7·3) for determination of respiration or (2) in 0·82 % Na_2CO_3 solution, neutralised with CO_2 gas, for measurement of glycolysis. In some cases the precipitate never entirely dissolved; this was due to the instability of the oxidation product, which in certain circumstances partially changes to an insoluble substance. Concentrations quoted below are therefore only approximate.

Measurement of metabolism.

The standard Warburg apparatus was used, with simple conical vessels with inset trough and side-arm. For respiration, 0·1 ml. of $2N$ NaOH was placed in the central trough, and the tissue in $M/15$ phosphate or Ringer-phosphate was contained in the main vessel. In some cases the oxidised dibenzanthracene solutions and substrate were also placed in the main vessel. When tissue slices were used it was however found advisable to measure the respiration in Ringer

alone for a certain time ($\frac{1}{2}$ to 1 hour) before adding the substrates and different concentrations of oxidation product from the side vessels. The reason for this was that variations up to 15 % occurred in the respiration of tissues from any one animal. Brain, and tumours containing much blood or necrotic material, showed variations outside these limits. The respiration was in all cases measured in oxygen which was passed through the vessels for 20 seconds before closing the taps. Tissues were minced finely with scissors and 0·1 or 0·2 g. suspended in $M/15$ phosphate in the Warburg vessels, or slices were prepared according to Warburg [1923] and the dry weights determined at the end of the experiment. In each set of experiments there was one thermobarometer, one vessel containing a sample of tissue in Ringer-phosphate (or phosphate) alone and another the same with lithium lactate or sodium succinate (final concentration $M/100$). The remaining vessels contained in addition varying concentrations of oxidised dibenzanthracene.

A typical experiment was as follows:

		Side vessel			
Vessel No.	Main vessel Ringer-phosphate ml.	Lactate ml.	Oxidised dibenzanthracene in phosphate ml.	Phosphate ml.	Inset 2*N* NaOH ml.
1	1	—	—	—	—
2	0·6	—	—	0·3 +0·1 H_2O	0·1
3	0·6	0·1	—	0·3	0·1
4	0·6	0·1	0·1	0·2	0·1
5	0·6	0·1	0·2	0·1	0·1
6	0·6	0·1	0·3	—	0·1

The vessels after being filled with oxygen were shaken for 15 minutes in the thermostat to reach temperature equilibrium, and then readings were taken every 10 or 15 minutes, generally for an hour, before tipping in the solutions from the side vessels. When minced tissue was used it was found unnecessary to determine the normal respiration as there were negligible differences between duplicate experiments; in these cases all the solutions were from the beginning in the main vessels with the tissue. For determination of glycolysis, the tissue was suspended in Ringer-bicarbonate containing glucose, and the dibenzanthracene derivative was dissolved in bicarbonate. A mixture of 95 % nitrogen and 5 % carbon dioxide was passed through the vessels for a minute at the beginning of an experiment.

A few sets of experiments on respiration and glycolysis were carried out under aseptic conditions, and cultures were made at the end of the experiment to test the sterility. In no case did the comparison of these and other experiments show differences which could be ascribed to bacterial contamination.

Results.

A. *Normal tissues.*

Muscle. Minced voluntary muscle of the rat in $M/15$ phosphate was used for the determination of respiration and anaerobic glycolysis at 25°. In one series of experiments on respiration, diaphragm muscle (rat) at 37·5° in Ringer-phosphate was used.

The tables show that a concentration of 0·02 % of oxidised dibenzanthracene reduced the oxygen consumption by a third in the first hour, and 0·3 % by 55 %.

Table I. *Minced rat muscle in M/15 phosphate* (p_H *7·3) at 25°.*

	Q_{O_2} Concentration of oxidised dibenzanthracene			
Hour	0	0·01 %	0·02 %	0·08 %
1	37·5	31·0	25·0	20·2
2	18·7	17·5	11·2	7·2
3	7·0	7·5	4·0	4·0

Table II. *Minced rat muscle in Ringer at 25°.*

	Q_{O_2} in Ringer-phosphate Concentration of oxidised dibenzanthracene		
Hour	0	0·1 %	0·3 %
1	25·0	14·3	11·3
2	20·5	9·5	4·0
3	12·8	7·5	2·0

	$Q_M^{N_2}$ in Ringer-bicarbonate Concentration of oxidised dibenzanthracene			
Hour	0	0·1 %	0·2 %	0·3 %
1	21·3	18·8	20·0	4·6
2	7·5	6·3	3·8	0·0

Table III. *Rat diaphragm in Ringer-phosphate at 37·5°.*

	Q_{O_2}			
Hour				
1	11·6	11·5	12·5	10·0
	Concentration of oxidised dibenzanthracene			
	0	0·03 %	0·06 %	0·09 %
2	9·7	9·0	7·7	5·7
3	8·5	8·6	5·8	5·2
4	7·9	7·5	5·4	3·8
	% decrease calculated on 1st hour			
2	16	22	38	43
3	27	25	54	48
4	33	35	57	62

In 3 hours this concentration almost stopped respiration and had completely arrested glycolysis within 2 hours.

As the glycolysis of minced rat muscle was inhibited by the oxidised 1:2:5:6-dibenzanthracene, a few experiments on the effect of the substance on glycolysis of rat muscle extract were carried out, with the co-operation of Dr C. A. Mawson. The muscle extract was made according to the method used by Meyerhof [1930]; although Meyerhof states that rat muscle yields an inactive extract Mawson (unpublished results) has been able to obtain glycolytically active rat muscle extracts. The results of experiments on undialysed and dialysed extracts showing pronounced inhibition of the lactic acid formation are given in Tables IV (*a*) and (*b*).

Table IV (*a*). *5 ml. undialysed rat muscle extract incubated 2 hours at 37°.*

			Oxidised 1:2:5:6-dibenz-anthracene %	mg. lactic acid	% inhibition of lactic acid formation
Unincubated				9·76	
Incubated with adenylpyrophosphate + $MgCl_2$				10·13	
,,	,,	+glycogen		17·64	
,,	,,	,,	+0·002	17·37	3·6
,,	,,	,,	+0·004	16·05	21
,,	,,	,,	+0·012	11·85	77

Table IV (*b*). *5 ml. rat muscle extract dialysed for 3 hours at 0°, incubated 2 hours at 37°.*

			Oxidised 1:2:5:6-dibenz-anthracene %	mg. lactic acid	% inhibition of lactic acid formation
Unincubated extract				1·34	
Incubated with adenylpyrophosphate and $MgCl_2$				1·66	
,,	,,	+glycogen		5·12	
,,	,,	,,	+0·002	4·39	21
,,	,,	,,	+0·004	3·27	54
,,	,,	,,	+0·012	2·37	78

Liver. All samples in the following tables, except those marked "No lactate" (or "No succinate") had an addition of $M/100$ lactate (or $M/100$ succinate) after an hour.

Table V. *Rat liver slices in Ringer-phosphate at 37°.*

	Q_{O_2}				
		Concentration of oxidised dibenzanthracene			
Hour	No lactate 0	0	0·002 %	0·005 %	0·01 %
1	4·3	7·3	7·5	6·2	3·4
2	3·4	6·6	6·5	5·0	2·0
3	3·3	6·2	5·0	4·7	1·6

Table VI. *Rat liver slices in Ringer-phosphate at 37°.*

Hour	Q_{O_2}				
1	9·3	9·1	8·2	9·3	8·3
		Concentration of oxidised dibenzanthracene			
	No lactate 0	0	0·1 %	0·2 %	0·3 %
2	9·3	10·0	8·6	9·8	7·1
3	8·5	8·7	8·1	8·9	6·2
4	7·8	8·1	7·5	7·6	5·7
	% difference, calculated on 1st hour				
2	0	+10	+5	+5	−14
3	−8	−4	−1	−4	−25
4	−16	−11	−8	−18	−31

Tables V and VI are typical of ten experiments on liver. Table V shows that the respiration was increased 70 % by the addition of lactate, and that the dibenzanthracene derivative greatly reduced the oxygen uptake. In the experiment represented by Table VI, on the other hand, the respiration was very slightly increased by lactate, and the inhibition was also less. The mean of six sets of experiments shows almost equal increase of respiration due to added lactate, and decrease due to the presence of oxidised dibenzanthracene, indicating that it is mainly lactic oxidation which is inhibited.

Kidney. Sections from rat kidney cortex were used to investigate the effect of the oxidised hydrocarbon on lactate and on succinate oxidation. As the normal respiration of kidney is higher than that of liver thinner sections (0·15–0·3 mm.) were used.

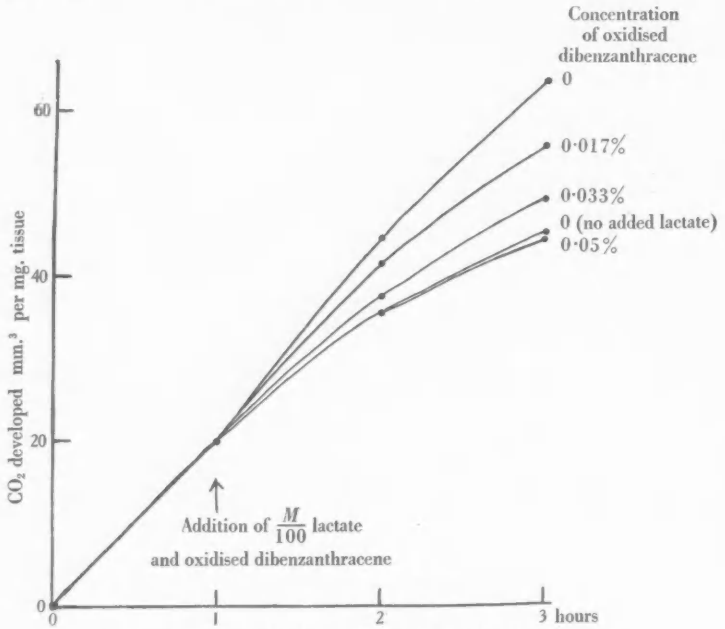

Fig. 1. Respiration of kidney slices in Ringer-phosphate at 37·5°.

Table VII. *Rat kidney slices in Ringer-phosphate (with lactate) at 37·5°.*

Hour	Q_{O_2}								
1	20·7	19·0	21·5	22·5	20·0	22·5	22·6	25·5	21·9
	No lactate	Concentration of oxidised dibenzanthracene							
	0	0	0	0·005 %	0·01 %	0·02 %	0·05 %	0·1 %	0·2 %
2	16·2	26·5	25·5	28·0	23·5	23·0	22·2	25·0	17·8
3	8·3	17·2	20·0	20·2	16·5	16·8	15·0	17·5	12·2
	% difference calculated on 1st hour								
2	−22	+40	+19	+22	+17	+2	−2	−2	−19
3	−60	−9	−7	−11	−17	−25	−34	−31	−45

Table VIII. *Rat kidney slices in Ringer-phosphate (with succinate) at 37·5°.*

Hour	Q_{O_2}				
1	16·8	18·0	17·8	15·5	16·8
		Concentration of oxidised dibenzanthracene			
	No succinate	0	0·017 %	0·033 %	0·05 %
2	13·9	31·5	25·1	18·8	21·8
3	8·8	16·5	15·0	11·2	11·8
	% difference, calculated on 1st hour				
2	−17	+75	+41	+21	+30
3	−48	−8	−16	−28	−30

Tables VII and VIII show that a concentration of 0·02 % of the benzanthracene derivative has a measurable effect on lactate oxidation. Table VII shows that within the experimental error increasing concentrations from 0·001 up to 0·2 % show a steadily increasing effect. A concentration of 0·033 % has an inhibitory effect on succinic oxidation.

Brain. A modification of the technique used by Quastel [1932] was employed in a few sets of experiments on minced rat's brain. The tissue was allowed to respire in phosphate solution for 2 hours before the lactate and the solutions of oxidised dibenzanthracene were added. The respiration fell rapidly during the first hour and then became more uniform, though still falling slowly. The normal respira-

Table IX. *Rat brain minced in M/15 phosphate (dry weights taken as 1/5 wet weights) at 37·5°.*

Hour	Q_{O_2}				
2	19·2	17·7	17·0	18·0	17·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·005 %	0·01 %	0·02 %
3	11·1	18·5	21·2	17·9	16·5
4	8·1	16·8	19·8	17·2	11·8
	% difference, calculated on 2nd hour				
3	−42	+4	+25	−1	−5
4	−58	−5	+16	−4	−33

Table X. *Rat brain slices in Ringer-phosphate (from dry weights, brain dried at 50°) at 37·5°.*

Hour	Q_{O_2}				
1	6·0	5·0	6·5	7·8	5·1
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	3·3	5·4	6·3	8·0	4·2
3	1·4	4·3	5·0	5·8	2·6
	% difference, calculated on 1st hour				
2	−45	+8	−3	+2	−18
3	−77	−14	−23	−25	−49

Table XI. *Rat brain slices in Ringer-bicarbonate (from dry weights, brain dried at room temperature) at 37·5°.*

	$Q_M^{N_2}$ Concentration of oxidised dibenzanthracene			
Hour	0	0·03 %	0·1 %	0·3 %
1	5·7	6·4	4·4	3·9
2	3·6	3·9	2·3	1·5

Table XII. *Rat brain slices in Ringer-bicarbonate (from dry weights, brain dried at room temperature) at 37·5°.*

	$Q_M^{N_2}$ Concentration of oxidised dibenzanthracene			
Hour	0	0·03 %	0·1 %	0·3 %
1	6·6	6·0	6·2	5·4
2	5·6	4·3	5·0	3·5

tion was taken as that during the second hour and the respiration in subsequent hours compared with it. Respiration and glycolysis were also investigated for brain slices in Ringer. As the glycolytic activity of brain decreased rapidly with time, the oxidised dibenzanthracene was in contact with the tissue from the beginning in the glycolysis experiments. Though this lessens the accuracy of the determinations the effect found was so large as to be outside the possible error. The sections were dried either at 50° or at room temperature in a desiccator. Tables IX–XII show that the minimum concentration to show an effect is about 0·02 %.

B. *Malignant tissues.*

Jensen's rat sarcoma (J.R.S.), and C 63[1], a transplantable mammary carcinoma of the mouse, were used in this work. In addition three strains of transplantable tumours produced by pure hydrocarbons were studied, and their respiration and anaerobic glycolysis measured for the first time. These latter tumours which are spindle-celled sarcomas were produced here by subcutaneous injection of solutions in lard of 1:2:5:6-dibenzanthracene (tumour R 10, rat) or of 5:6-*cyclo*penteno-1:2-benzanthracene (tumour M 154, mouse), or of 1:2:5:6-dibenzanthracene in lard and milk (tumour M 4, mouse). The first of these has now reached the 51st, the second the 44th and the third the 83rd grafted generation.

Jensen's rat sarcoma. In addition to experiments similar to those described for other tissues, in a few cases considerable precautions were taken to avoid bacterial infection. The solutions used were all sterilised and measured out from sterile pipettes, the Warburg vessels and Petri dishes were autoclaved and the tumours removed aseptically from the rats. Sterile finger-stalls were used when cutting the sections. The gases were passed through cotton wool plugs, and the parts of the manometer through which they passed were rinsed out with absolute alcohol. Cultures were taken from the liquids at the end of the experiments and examinations made after 18 hours' incubation. Table XIV shows the result of one such experiment, with the bacteriological report.

[1] We are indebted to the Imperial Cancer Research Fund for this tumour.

Table XIII. *J.R.S. slices at 37·5°.*

Hour	Concentration of oxidised dibenzanthracene: 0	0·1 %	0·2 %	0·3 %
	Q_{O_2} in Ringer-phosphate			
1	12·2	8·6	9·8	8·4
2	8·8	8·6	9·0	7·3
3	7·0	7·4	6·6	5·0
	$Q_M^{N_2}$ in Ringer-bicarbonate			
1	26·2	18·5	15·1	21·0
2	19·0	17·4	11·7	11·4
3	14·4	12·8	5·3	5·8

Table XIV. *J.R.S. slices at 37·5°, aseptic technique.*

(Roman numerals refer to bacteriological report.)

Hour	Q_{O_2} in Ringer-phosphate: No lactate I	Concentration of oxidised dibenzanthracene: II 0	III 0·1 %	IV 0·2 %	V 0·3 %
1	10·0	10·4	8·2	7·4	7·2
2	7·8	8·7	7·3	6·3	4·5
3	7·1	6·9	6·5	5·4	3·1

	$Q_M^{N_2}$ in Ringer-bicarbonate. Concentration of oxidised dibenzanthracene: VI 0	VII 0·1 %	VIII 0·2 %	IX 0·3 %
1	19·2	14·0	15·0	9·5
2	14·0	11·1	11·4	6·1
3	8·0	5·7	4·6	4·1

Sterility test on fluid from experiment shown in Table XIV after 18 hours' incubation.

Vessel I. Slight growth of streptococci and *B. subtilis.*
Vessel II. Moderate growth of staphylococci and streptococci.
Vessel III. Very slight growth of staphylococci.
Vessel IV. Moderate growth of streptococci and staphylococci.
Vessel V. Very slight growth of staphylococci.
Vessel VI. Very slight growth of *B. subtilis.*
Vessel VII. Sterile.
Vessel VIII. Sterile.
Vessel IX. Sterile.

Eighteen hours' incubation after the conclusion of the experiment constitutes a somewhat severe test of aseptic technique. It is evident in Table XIV that such degrees of contamination as had occurred show no parallelism with the chemical changes measured (*e.g.* vessels III, IV, V).

Dickens [1931] quotes results for J.R.S.:

Warburg, $Q_{O_2}=9$, $Q_M^{N_2}=34$;
Crabtree, $Q_{O_2}=8$, 11, $Q_M^{N_2}=32$.

Rat sarcoma 10. Tables XV and XVI, typical of experiments on this tumour, show that concentrations of less than 0·1 % oxidised dibenzanthracene produce

no inhibition of oxygen uptake, within the times of the experiments (2–3 hours) and that higher concentrations (0·2, 0·3 %) show an effect only after one hour.

Table XV. *R. 10 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	12·5	12·2	12·6	13·0	13·2
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	12·2	12·2	12·6	11·5	13·5
3	11·1	11·7	10·7	10·7	12·5
	% difference, calculated on 1st hour				
2	−2	0	0	−12	+2
3	−11	−4	−15	−18	−5

Table XVI. *R. 10 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}			
1	13·8	15·0	12·7	13·8
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	12·4	13·4	11·4	11·4
3	10·4	7·9	4·8	3·5
4	10·0	6·7	2·7	2·1
	% difference, calculated on 1st hour			
2	−11	−11	−10	−17
3	−25	−47	−62	−75
4	−28	−55	−80	−85

Table XVII. *R. 10 slices in Ringer-bicarbonate at 37·5°.*

Hour	$Q_M^{N_2}$			
1	19·0	18·3	22·3	22·0
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	14·5	13·8	12·0	10·0
3	11·6	11·7	9·2	5·7
4	6·2	7·1	6·2	4·2
	% difference, calculated on 1st hour			
2	−23	−25	−45	−55
3	−39	−35	−57	−74
4	−67	−61	−73	−82

Mouse sarcoma M 154. It was difficult to get sections of this tumour completely free from necrotic material, and as the method of calculation used makes use of the absolute value of the initial respiration of each sample, no great trouble was taken to ensure the absence of necrosis from the slices used. This accounts for the considerable differences in amount of respiration found. It will be seen that in the glycolysis experiments the differences in the first hour (after adding the oxidised dibenzanthracene) are greater than could reasonably be accounted for by the presence of some necrotic material.

Table XVIII. *M 154 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	10·0	9·8	9·5	8·4	8·0
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	7·3	9·6	7·8	8·0	7·4
3	5·8	7·5	6·5	6·3	5·0
4	3·7	6·7	5·4	5·4	4·3
5	3·1	5·8	5·1	4·7	2·6
		% difference, calculated on 1st hour			
2	−27	−2	−18	−5	−8
3	−42	−23	−31	−25	−38
4	−63	−32	−43	−35	−47
5	−69	−41	−46	−44	−68

Table XIX. *M 154 slices at 37·5°.*

Hour	Q_{O_2} in Ringer-phosphate			
1	11·5	12·1	8·6	9·3
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	11·0	9·7	5·2	1·8
3	9·1	8·4	2·8	1·5
	% difference, calculated on 1st hour			
2	−4	−20	−40	−81
3	−21	−30	−68	−84
	$Q_M^{N_2}$ in Ringer-bicarbonate			
1	21·5	12·8	10·6	7·8
2	18·4	12·2	8·3	6·2
3	17·4	10·3	6·7	5·7

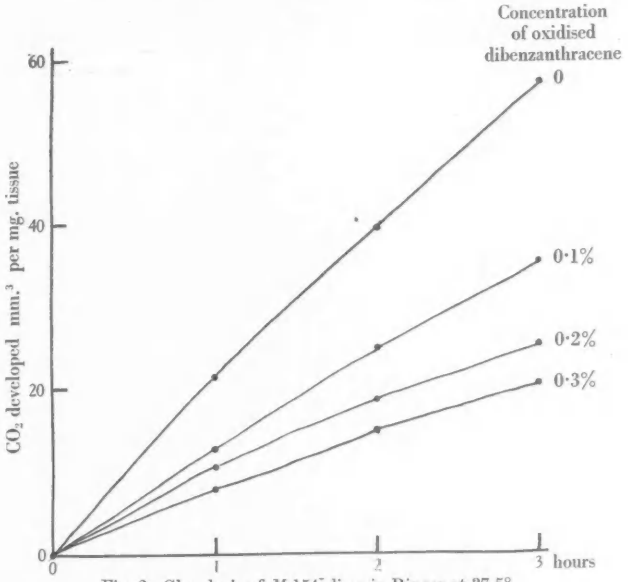

Fig. 2. Glycolysis of *M* 154 slices in Ringer at 37·5°.

Mouse sarcoma M 4. Table XX shows one of three similar experiments on M 4. A concentration of 0·033 % produced an inhibitory effect within 3 hours and 0·05 % after one hour. It is thus rather more susceptible than R 10 and J.R.S.

Table XX. *M 4 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	9·0	10·6	9·5	9·0	10·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	8·4	9·9	8·9	9·0	9·7
3	7·1	8·5	7·9	7·5	7·0
4	6·2	7·2	6·1	5·0	3·8
	% difference, calculated on 1st hour				
2	−7	−6	−6	0	−8
3	−20	−20	−17	−17	−33
4	−31	−32	−36	−45	−64

Mouse carcinoma C 63. This tumour is vascular and has a small growing edge of sound tissue; sections were therefore difficult to cut free from necrotic material. This tumour appears to be rather more susceptible to the action of the oxidised dibenzanthracene than the others studied, as inhibition of respiration is observable after one hour even with a 0·033 % solution and 0·1 % produces almost the maximum inhibition (Tables XXI–XXIII).

Table XXI. *C 63 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	8·0	11·5	8·3	10·6	12·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	6·7	10·5	7·2	7·2	8·0
3	5·4	8·2	6·6	6·4	6·7
4	3·7	6·8	4·7	4·5	4·8
	% difference, calculated on 1st hour				
2	−16	−9	−13	−32	−36
3	−32	−29	−20	−40	−46
4	−54	−41	−43	−58	−62

Table XXII. *C 63 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	18·0	12·8	15·2	11·2	17·6
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·1 %	0·2 %	0·3 %
2	10·2	8·6	8·0	5·3	7·5
3	7·4	5·5	4·8	2·8	3·8
	% difference, calculated on 1st hour				
2	−43	−33	−47	−53	−56
3	−59	−57	−70	−74	−78

Table XXIII. *C 63 slices in Ringer-bicarbonate at 37·5°.*

Hour	$Q_M^{N_2}$			
1	15·5	19·4	12·2	14·3
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	18·2	16·1	8·4	8·5
3	15·7	10·2	6·4	5·2
	% difference, calculated on 1st hour			
2	+17	−17	−31	−41
3	+1	−47	−48	−63

Conclusion.

The tables given above show that oxidised 1:2:5:6-dibenzanthracene acts as an inhibitor of oxidation and glycolysis in all the tissues studied. There is no remarkable difference between the effects on normal and on malignant tissues, except that the malignant tissues with the exception of C 63 are more slowly inhibited. A concentration of 0·2 % in every tissue examined produces a measurable diminution of metabolism; in the case of kidney and muscle 0·02 % is effective. Boyland [1933], using the methylene blue technique, found that a 0·002 % solution of the hydrocarbon produced after oxidation more than 50 % inhibition of lactic dehydrogenase from yeast, but the quantity of this enzyme present was probably much smaller than in the present experiments.

As all known reactions involving enzymes take place in aqueous solution, it seems probable that carcinogenic hydrocarbons are altered in the body to form water-soluble compounds before they can produce biological effects. The oxidation products of these hydrocarbons produced by oxygen in ultra-violet light are water-soluble and have a large inhibitory effect on oxidation processes. The present work shows that the action of the oxidised 1:2:5:6-dibenzanthracene on malignant cells is very similar to that on normal tissues. It is possible that the production of tumours by these carcinogenic compounds involves an oxidation of the compound, either to a product identical with that isolated and used in the foregoing experiments, or to a less stable intermediate oxidation product. Subcutaneous injections of 0·4 % solutions of the oxidation products used in the present work have not yet, after 14 weeks, produced any tumours in rats or mice.

Summary.

The effect of a water-soluble oxidation product of 1:2:5:6-dibenzanthracene on the metabolism has been studied in respect of the following rat tissues: 1. Muscle, respiration and glycolysis: 2. Liver, respiration: 3. Kidney, respiration: 4. Brain, respiration and glycolysis: 5. Jensen's sarcoma and rat sarcoma 10, respiration and glycolysis; and of the following mouse tissues: 6. Sarcoma M 154, respiration and glycolysis: 7. Sarcoma M 4, respiration: 8. Carcinoma 63, respiration and glycolysis.

In all cases an inhibitory effect was found.

Our thanks are due to Dr C. C. Bryson, Assistant Pathologist to the Cancer Hospital, for his bacteriological investigations on the samples submitted to him; and also to Prof. Kennaway for his interest and encouragement during the course of the work here described.

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XXXIV. THE METABOLISM OF GALACTOSE.

II. THE BEHAVIOUR OF THE RAT TOWARDS MODERATE AMOUNTS OF GALACTOSE.

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(*Received January 3rd, 1934.*)

The immediate purpose of the work reported in this and the previous paper [Harding and Grant, 1932–33] has been to trace as far as possible, by methods of direct analysis, the fate of orally administered galactose. The use of galactose-acclimatised yeast [Harding and Grant, 1931–32] or *S. marxianus* [Harding, Nicholson and Grant, 1932–33] as an analytical reagent enables free galactose in extracts or preparations of blood, urine and tissues to be estimated with precision. The previous paper showed that the oral administration of moderate amounts of galactose (40 g.) to adult man resulted in a rapid entrance of galactose into the blood stream, with a peak amount at 30–60 mins. followed by a fall to zero by the end of 2 hours. A small increase in arterial blood-glucose was also noted. The urine contained amounts not greater than 2 g. galactose. With this amount of galactose, all the sugar was disposed of in 2 hours, but with a larger dose (80 g.) the excretion into the urine sometimes continued until the fourth hour. No evidence was found in the blood or urine of any new reducing compound formed from galactose, nor did the urine show any trace of non-reducing substance which yielded galactose on hydrolysis, beyond those small, and as yet doubtful, amounts demonstrated in the fasting condition by Harding and Grant [1931–32]. The present communication traces the absorption of galactose from the intestine of the rat and its disappearance from the tissues.

Experimental and analytical methods.

Young adult white rats (both sexes) were starved for 24 hours. To Series A was given by stomach tube [Cori, 1925] 1 ml. of 10 % galactose solution. The rats were placed in a cage with provision for urine collection. Five minutes previous to the desired time 15–20 mg. amytal were injected. At the end of 5, 20, 60 or 180 mins. from the administration of the galactose, the alimentary tract was ligatured at the oesophagus and anus and removed. Any urine was removed from the bladder by a small syringe, the bladder being washed out 3 or 4 times with distilled water. The muscle specimen, if desired, was taken from the hind-leg by dissecting away the skin, freezing by solid CO_2 *in situ* and then removing. The liver, if desired, was removed and placed at once in solid CO_2. If the whole animal was desired, it was frozen by solid CO_2 immediately after the removal of the urine. In a few instances a blood specimen was taken from the tail previous

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to the freezing. To Series B was given 1 ml. of 20 % galactose solution, and the animals were examined at the end of 20, 60, 120 and 180 mins. A few control animals were examined at the end of 20 mins.

Preparation previous to analysis. The excised alimentary tract was at once opened throughout its entire length and washed out with distilled water three or four times, gently macerating the interior lining. The extract was treated with 10 % sodium tungstate and 2/3 *N* H_2SO_4. After standing for a few minutes this was filtered. The filtrate was shaken with purified Lloyd's reagent [Hamilton, 1928] and refiltered. The frozen muscles were weighed, dropped into 15 ml. of boiling water and extracted for 15 mins. The solution was decanted and the residue ground in a mortar and re-extracted with 10 ml. of boiling water for a second 15 mins. interval. The solution was again decanted and the residue placed in a test-tube and extracted on a boiling water-bath with three successive portions of 10 ml. of hot water, taking 30 mins. for the final extraction. The residue was strained with pressure through a piece of cheese cloth. The total cooled extract was made up to 60 ml. The frozen liver was extracted in exactly the same manner as the muscle tissue, except that the residue was separated by centrifuge.

When an examination of the whole animal (*minus* intestinal contents and urine) was required, the frozen carcass was minced, placed in 100 ml. of boiling water and extracted for 15 mins. The solution was centrifuged and the residue re-extracted with three successive portions of boiling water (100 ml.), the last extraction interval being 30 mins. The cooled extract was made up to 500 ml.

To 60 ml. of the tissue extract were added 15 ml. of 10 % $ZnSO_4, 7H_2O$ solution and 15 ml. of 0·5 *N* NaOH solution, gradually with shaking of the flask. The precipitates from the muscle and liver extracts were filtered, using an ordinary funnel. The whole animal filtrate was obtained by suction through a Büchner funnel of wide area. It was later found more convenient to precipitate 120 ml. of the whole animal extract, especially in the case of larger animals, as the filtration was slow owing to the presence of fat. In such cases there was occasionally some difficulty in obtaining a clear filtrate. 4·5 g. of purified Lloyd's reagent were added to 50 ml. of the zinc filtrate and the mixture shaken for 2 mins. and filtered. It was found necessary to make this filtrate alkaline with two drops of 40 % NaOH solution to precipitate the excess zinc present. Any excess zinc was found to interfere with the sugar oxidation and also with the removal of the sugars by yeasts. The precipitate of $Zn(OH)_2$ was filtered off, and the filtrate was adjusted to p_H 6–6·5, with 10*N* H_2SO_4.

The above details form a slight modification of Somogyi's [1930] directions for the preparation of blood-filtrates suitable for sugar analysis. We were unable to prepare suitable filtrates from tissue by the use of tungstic acid [Power and Clawson, 1928].

Analysis for glucose and galactose. The final clear filtrates from the alimentary tract, muscle, liver or whole animal were analysed for glucose (fermentable sugar) and galactose by the method of Harding and Grant [1931–32]. A double action of ordinary baker's yeast and galactose-acclimatised yeast[1], followed by a double centrifugation previous to the determination of the reducing power, ensured the complete removal of the desired sugar at each stage of analysis. The reducing power was determined by a modified Shaffer-Somogyi reagent [Harding and Downs, 1933]. Galactose in the blood specimens was determined by the method of Grant and Glaister [1933] which is suitable for the estimation of non-fermentable sugar in blood. This method of determining galactose in presence

[1] The authors wish to thank the Standard Brands Inc. New York for the continued preparation of "galac" yeast.

of glucose and the reducing substances in blood corpuscles has been later employed by Harding *et al.* [1933] in the determination of blood-xylose. Urine was analysed according to the previous directions of Harding and Grant.

Recovery of added galactose. Galactose added to muscle, liver or whole animal hash was recovered in 98–105 % yield; added to intestinal contents the recovery yield was 80–90 %.

Results and Discussion.

The concentrations of glucose, galactose and residual reducing substances together with the total amounts of galactose found in the alimentary tract, urine and whole animal are shown in Tables I (Series A) and II (Series B).

Table I. *Determinations in whole animals of concentrations of tissue "sugars" in individual control rats, and average galactose values at stated intervals after the ingestion of approximately 0·6 g. galactose per kg. body weight (Series A).*

Rat					Tissue "sugars" (mg./100 g.)			Galactose distribution. Total amount in mg. (as galactose)			
No.	Wt. (g.)	Dose mg.	Time mins.	Blood-galactose mg./100 ml.	Glucose	Galactose	Residual reduction (as glucose)	Alimentary tract	Urine	Tissues (as free sugar)	Utilised
51	—	—	—	—	60	4	30	—	—	—	—
52	—	0	0	0	38	2	18	0	—	—	—
53	—	—	—	—	41	(1)	18	—	—	—	—
54	—	—	—	—	55	(1)	24	—	—	—	—
				Average	48	2	22				
Average of 4	143	92	5	0	39	3	19	76	—	6	10
„ 7	162	90	20	77	41	19	20	40	2	29	17
„ 8	153	89	60	44	44	25	23	4	15	34	36
„ 5	147	94	180	11	49	3	24	0	25	4	64

Table II. *Average concentrations of tissue "sugars" and total amounts of galactose in whole animals after ingestion of approximately 1·2 g. galactose per kg. body weight at end of stated intervals (Series B).*

	Average				Tissue "sugars" mg./100 g.			Galactose distribution. Total amount in mg. (as galactose)			
No. of exps.	Rat wt. (g.)	Dose mg.	Time mins.	Blood-galactose mg./100 ml.	Glucose	Galactose	Residual reduction (as glucose)	Alimentary tract	Urine	Tissues (as free sugar)	Utilised
6	156	184	20	86	39	21	16	119	3	32	30
6	134	180	60	126	49	33	19	12	42	82	45
5	148	185	120	57	47	23	21	3	75	34	73
6	150	191	180	20	58	6	20	(1)	76	9	105

At the outset we wish to emphasise the relatively small dose of galactose we have given. Series A received an average of 0·6 g. per kg. and Series B, 1·2 g. per kg. These amounts are comparable with the feeding of 40 and 80 g. galactose to the adult man. True, even these amounts are much in excess of what either the rat or man would receive under natural conditions, but they are much less than those administered by Cori [1925], who gave 8–9 g. per kg. by mouth to the rat, or the 2 g. per kg. per hour given intravenously by Wierzuchowski *et al.* [1931] to the dog. The dose given by Cori to the rat is equivalent to 500–600 g. galactose given to man.

Absorption from the intestine. Both in Series A and B, the absorption of galactose is practically complete at the end of an hour. Galactose is known to be rapidly absorbed in the dog [Nagano, 1902], cat [Hewitt, 1924], rabbit [Hédon,

1900], and rat [Cori, 1925]. Mainly from a study of glucose absorption, but stating that the figures on the absorption of other sugars support the same conclusion, Cori has formulated laws of absorption of sugars from the intestine. "The rate of absorption of a sugar is independent of the amount or concentration of the sugar fed." These conclusions for the absorption of glucose have been questioned by other observers [MacKay and Bergman, 1933]. Cori's absorption rate for galactose is 1·96 [1925] and 1·82 [1926] g. per kg. per hour. From his average figures it can be seen that at the end of 3 hours there were left unabsorbed 1·31, 3·47 and 3·94 g. galactose per kg. body weight. Only the first is comparable with our highest amounts fed in Series B. In Table III we show the

Table III. *Rate of absorption of galactose over short periods of time when given in moderate amounts.*

	After 20 mins. Amount absorbed		After 60 mins. Amount absorbed
	mg./100 g.	mg./100 g. per hr.	mg./100 g. per hr.
Series A	31	93	55
Series B	42	125	125

absorption rates (calculated from the data of Tables I and II) of galactose when administered in amounts below those investigated by Cori. In no case does the absorption rate approach 1·82 g. per kg. per hour. Our figures are few but the most significant give a value of 1·25 g. per kg. per hour. The very short time of our absorption periods, however, may introduce an error tending to low results, but in Series B the rate in the first 20 minutes is the same as in the next 40 minutes. Other differences in the experimental conditions are to be noted. Our animals were starved for 24 hours previous to the feeding, Cori's for 48 hours. By analogy with the absorption of glucose and fructose this should raise our rates of absorption rather than decrease them [Cori and Cori, 1927]. Amytal anaesthesia also lowers the rate of absorption about 30 % [Cori, 1931]. Only a few minutes elapsed, however, between the administration of the amytal and the excision of the alimentary tract. Nor did we divide the sugar remaining in the alimentary tract between the stomach and intestine. Any galactose left in the former would of course raise the calculated rate of absorption from the intestine. McCance and Madders [1930] and Trimble *et al.* [1933] have pointed out the part taken by the pyloric discharge in determining rates of intestinal absorption. The subject of absorption of sugars at low initial concentrations invites further investigation.

Fermentable sugar was also found in irregular amounts in the intestinal contents. It is to be noted that both in Series A and B, at the end of 3 hours, when galactose was still present in the blood stream, none was present in the alimentary tract.

Galactose in the tissues. In our control animals the examination of individual muscle or whole animal always showed the presence of small amounts of substance removable by "galac" yeast. This was not removable by the two previous treatments with ordinary baker's yeast, and while the first treatment with "galac" yeast always effected a small removal the second treatment failed to show any further removal. The amounts varied from 1 to 6 mg./100 g. tissue or muscle (Tables I and IV). Such amounts are just at, or very slightly above, our experimental error. Consequently we cannot place too much reliance on them. Liver extracts showed no evidence of such a removal, but these extracts were much more dilute. No free galactose was found in control rat blood or urine,

Table IV. *Concentrations of muscle and liver "glucose," galactose and residual reducing substances one hour after ingestion of 190 mg. galactose by the rat.*

	mg./100 g.					
	Muscle			Liver		
	Glucose	Galactose	Residual reduction (as glucose)	Glucose	Galactose	Residual reduction (as glucose)
Controls	25	0	4	—	—	—
	34	0	5	—	—	—
	26	2	4	101	0	21
	32	6	8	114	0	19
	38	3	7	162	0	20
	33	2	5	97	0	21
	22	2	9	80	0	17
Average	30 ±6	2·5 ±2	6 ±2	111 ±20	0	18 ±3
One hour after galactose	34	33	7	98	104	17
	33	27	14	129	129	25
	35	36	10	144	111	20
	38	33	9	97	130	35
	33	37	5	143	98	21
Average	35 ±2	33 ±4	9 ±3	122 ±21	114 ±14	23 ±7

nor did Harding and Grant find any in normal human blood and urine. However, the dilutions of 1 : 10 used by them would fail to show amounts less than 2–3 mg./100 ml. In view of the fact that free galactose is readily excretable both in man and the rat it seems unlikely that our analytical finding represents galactose as a free constituent of living muscle.

Corresponding to the rapid absorption of galactose from the intestine there is a rapid rise of this sugar in the blood and tissues. Our observations are too infrequent for us to do more than state that the maximum concentration of galactose in the tissues and blood occurs somewhere within or about the first hour, with either the smaller or larger dose. This same maximum in the blood occurs in man. Are we to infer that the absorption of galactose from the intestine of man is as rapid as in the rat?

At the end of 3 hours after the smaller dose, the galactose content of the whole animal has fallen to the low indefinite figure of the control. After the larger dose, it is sufficiently low (4–9 mg./100 g.) to enable us to state that all but traces of the ingested sugar have disappeared.

Fermentable sugar, residual reducing substances and glycogen after galactose. Our method of analysis gives us, in addition to galactose, the concentration of glucose (fermentable sugar) and residual reducing substances. Kosterlitz and Wedler [1933] examined the sugar of the liver after galactose ingestion. They found slight increases in fermentable sugar but not in such amount as to lead to any definite conclusion. Their residual reducing fraction, which included galactose, was increased. Examination of our whole animal experiments shows a slight average increase in "glucose" at the end of the higher dose experiments, but in view of the large variations little importance can be attached to the figures. Harding and Grant found increases in blood-glucose in man after galactose feeding. The normal variations in blood-glucose are small, however, and experimental fluctuations are thus more easily ascertained. Certainly, in the rat, there occurs no transformation of ingested galactose into glucose sufficiently rapid to cause an accumulation of this latter sugar. In an endeavour to check more strictly the possible occurrence of rapid galactose-glucose conversion, we

examined a section of leg muscle and the liver, one hour after the feeding of the higher dose of galactose. The results are shown in Table IV. The increase in the concentration of galactose is plain in both muscle and liver; but again, the big variation in the "glucose" prevents any definite conclusion. Our figures for "fermentable" sugar in rat muscle (30 ± 6 mg./100 g.) are much higher than those recently published by Cori *et al.* [1933] (10–12 mg./100 g.), though these workers obtained much higher figures for the resting muscle of the amytalised animal. They agree more nearly with those of Power and Clawson [1928] and Bischoff and Long [1932]. The occurrence of any extensive glycogenolysis would impair the value of any experiments designed to measure an increase of that sugar after galactose feeding. Our animals were extremely quiet, however, and our muscle freezings and excisions were rapid. It is interesting to note that our figures for the non-fermentable sugar fraction of muscle extract obtained with $Zn(OH)_2$ as a precipitating agent followed by Lloyd's reagent are lower (4–9 mg./100 g.) than those of Cori *et al.* [1933] (8–22 mg./100 g.), who used $HgSO_4$–$BaCO_3$, though two of our animals after galactose gave 10 and 14 mg./100 g.

The constancy of the concentration of residual reducing substances indicates the thoroughness of our methods of extraction and sugar analyses. It indicates also the improbability of any conversion of galactose into another extractable reducing carbohydrate at such a rate that accumulation occurs. It may be noted that Bischoff and Long [1932] claim to have proved the absence of hexose-phosphate from muscle extracts precipitated by $Zn(OH)_2$. Harding and Grant noted also in man the absence of any new reducing carbohydrate in blood and urine after administration of galactose. Bierry *et al.* [1932] have claimed that they have proved the occurrence in liver of a galactose-containing polysaccharide. We did not examine the hydrolysis products of our animal or muscle hash after extraction to see if any combined or non-extractable galactose occurred. We, however, examined both liver- and muscle-glycogen in 2 rats 4 hours after the ingestion of 400 mg. galactose. The glycogen was extracted by the modification of Pflüger's method detailed by Good *et al.* [1933]. The glycogen obtained was hydrolysed and analysed for glucose and galactose. Only glucose was found.

Excretion into the urine. At the end of 20 minutes small amounts of galactose were found in the urine in both Series A and B. At the end of 3 hours 25 % of the smaller dose of galactose was found in the urine and 40 % of the larger. Cori [1926] found 60 % excretion in 4 hours. In both our series the excretion into the urine is greater than in man; 4 % excretion was the highest noted by Harding and Grant in man after the ingestion of 40 g. galactose, and 8 % after the ingestion of 80 g.

Conclusions.

The essential difference between the rat and man in their behaviour to moderate doses of galactose appears to be in the slower rate of utilisation by the rat. Parallels with other sugars are not wanting—thus the rat utilises intravenous glucose at a much more rapid rate than the dog or man [Cori and Cori, 1927]. The low rate of metabolism of intravenously administered galactose is also seen in the dog (0·2 g. per kg. per hour) [Wierzuchowski, 1931]. From Table II it can be seen that an average of 73 mg. galactose disappears in 2 hours from 150 g. body weight. This means an average utilisation of 0·25 g. per kg. per hour. After the corresponding dose to man Harding and Grant found a disappearance of over 90 % of the galactose in 2 hours—a disappearance rate of approximately 0·5 g. per kg. per hour. Whilst these figures, calculated as they are from experiments varying widely in conditions, must be taken as a very rough

approximation, they nevertheless support the idea that galactose utilisation in man is much better than experimental work on other animals has led us to anticipate. This low rate of change in the rat, coupled with the high rate of absorption and excretion, is responsible, according to Guha [1931], for the failure of this animal to grow or maintain life when galactose is the sole carbohydrate. In man and woman [Duel *et al.*, 1932; 1933] galactose is active as a nitrogen-sparing carbohydrate and an anti-ketogenic body. In this latter capacity it is even claimed that it is superior to glucose, its effect lasting 2 or 3 days. These two extreme statements may well be reconciled by a difference in the rate of transposition in different species.

SUMMARY.

Galactose, fed to rats at levels comparable with those at which it may be administered to man (0·6 g. and 1·2 g. per kg.), is almost completely absorbed from the intestine within an hour.

With both dosages of galactose, all but traces of the free sugar has disappeared from the tissues at the end of 3 hours.

There is proportionately a much larger excretion of the ingested galactose into the urine in the rat than in man. There thus appears to be a lower rate of conversion of galactose into its metabolites in the rat.

No evidence was found indicating the conversion of galactose into glucose or into any other reducing carbohydrate. The glycogen formed after galactose ingestion, either in the muscle or the liver, gives only glucose on hydrolysis.

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XXXV. THE CHEMOTHERAPY OF DERIVATIVES OF HARMINE AND HARMALINE. II.

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(*Received January 3rd, 1934.*)

In the first paper of this series, Coulthard *et al.* [1933] described the preparation of a long series of *O*-alkyl derivatives of harmol and recorded their bactericidal properties. It was shown that the peak compound of the normal alkyl series differed with the test organism, being situated at the butyl compound for *Bacillus typhosus* and at the amyl for *Staphylococcus aureus*, whilst it was recorded briefly that the most effective of the *n*-alkylharmols towards *Entamoeba histolytica* was *O*-*n*-nonylharmol. The experimental results upon which the latter statement was based are described in the present communication.

The organism chosen for our tests was *Entamoeba histolytica*, two strains of which, D.K.B. and N.R.S., were kindly supplied by Mr C. Dobell. Since a survey of the literature appeared to indicate that emetine was the most efficient lethal agent for this protozoon *in vitro*, this alkaloid was used as a control, but in addition tests were made upon some other substances used, or recommended for use, in the treatment of amoebic dysentery.

Laidlaw *et al.* [1928] described a method of testing emetine in which the test organism, *Entamoeba histolytica*, is cultivated in a medium consisting of Ringer's solution with horse-serum and a little rice starch, disodium hydrogen phosphate being added as a buffer. In this medium they found that the amoebae were destroyed in four days by emetine 1 in 5,000,000 provided that the medium did not become too acid. When the phosphate buffer was omitted, however, the end-point was not always sharp but varied from strain to strain and even with the same strain, the tubes which became most acid in culture being most likely to contain living amoebae. In these experiments the lethal concentration varied from 1 in 300,000 to 1 in 750,000.

In order to carry out our tests in such a manner that the minimum lethal concentration of our emetine control was relatively level from test to test, the medium buffered with phosphate was used in a series of determinations of the amoebicidal efficiency of the hydrochlorides of these harmine and harmaline derivatives. The results of this series of tests are given in Table I.

This table shows the gradual increase of the minimum lethal concentration of the *O*-*n*-alkylharmols from 1 in 80,000 (*O*-*n*-ethylharmol hydrochloride) to 1 in 200,000 to 1 in 500,000 for the *O*-*n*-nonyl derivative with subsequent decline for higher members of the series. The corresponding derivatives of harmalol are less efficient as are the *iso*-alkyl ethers of harmol.

The figure obtained for emetine is in good agreement with that (1 in 5,000,000) found by Laidlaw *et al.* [1928] using the same medium. By the courtesy of Dr T. A. Henry we obtained samples of *O*-methylpsychotrine sulphate and conessine acid oxalate, and brief reference to our results with these two salts may

Table I.

Compound	Minimum concentration lethal to *Entamoeba histolytica*
Hydrochlorides of:	
Harmol	1 in 40,000 to 1 in 80,000
O-Ethylharmol	1 in 80,000
O-*n*-Propylharmol	1 in 80,000 to 1 in 120,000
O-*n*-Butylharmol	1 in 20,000 to 1 in 80,000
O-*n*-Amylharmol	1 in 100,000 to 1 in 200,000
O-*n*-Hexylharmol	1 in 100,000 to 1 in 200,000
O-*n*-Heptylharmol	1 in 200,000
O-*n*-Octylharmol	1 in 200,000 to 1 in 300,000
O-*n*-Nonylharmol	1 in 200,000 to 1 in 500,000
O-*n*-Decylharmol	1 in 100,000
O-*n*-Dodecylharmol	1 in 100,000 not lethal
O-*iso*Propylharmol	1 in 80,000 not lethal
O-*sec*-Octylharmol	Less efficient than *O*-*n*-octylharmol
O-Allylharmol	1 in 80,000
O-Benzylharmol	1 in 80,000
Emetine hydrochloride	1 in 2,000,000 to 1 in 10,000,000
Conessine acid oxalate	1 in 80,000 to 1 in 200,000
O-Methylpsychotrine sulphate	1 in 80,000 not lethal

be made in passing. The very low result obtained with methylpsychotrine bears out the clinical results of Jepps and Meakins [1917] and the laboratory work of Dobell and Laidlaw [1926]. On the other hand, the figure obtained for conessine appears surprisingly low in view of the work of Brown [1922] and Henry and Brown [1923] on the action of conessine on free living protozoa, and the experiments of Chopra *et al.* [1927] on *Entamoeba histolytica* from kitten's faeces. A number of clinical successes have been reported with kurchi but it is interesting to note that conessine is regarded by some workers as less efficient than kurchi itself. [See, for example, Caius and Mhaskar, 1928; Knowles, 1928; Henry and Brown (quoting Willmore), 1928.] In support of our figure it may be noted that Knowles [1928] as the result of a clinical investigation suggested that the injection of conessine or total kurchi alkaloids was less efficient than kurchi orally, and Leake [1932] also considers kurchi ineffective, quoting the result of a clinical investigation by Reed using kurchi bismuth iodide.

It is evident from various papers, for example, that of Laidlaw *et al.* [1928] and that of Henry and Brown [1923], that the exceedingly high efficiency of emetine *in vitro* of the order of 1 : 5,000,000 is only found in alkaline, neutral or only very faintly acid media. Our results afford abundant confirmation of this fact. When endeavouring to assess the value of an amoebicide in the treatment of amoebic dysentery by comparison with emetine *in vitro* it appears therefore necessary to consider carefully the hydrogen ion concentration likely to be met with in the areas infested with amoebae.

We have been unable to find any reference to the actual hydrogen ion concentration in the amoebic ulcer, but Knowles *et al.* [1923] found that the p_H of a number of stools containing motile amoebae averaged 6·22. They also reported the results of experiments on kittens artificially infected with *E. histolytica*, in which the colon and rectum of the animals were minced in saline and the hydrogen ion concentration of the suspension determined. The average p_H value obtained in these experiments was 6·33, and the livers when similarly treated showed an average p_H value of 6·34.

Furthermore, a considerable amount of work has been carried out upon the reaction of living, dead and diseased body cells, and the work of Rohde [1927, 1, 2],

and Chambers *et al.* [1927] suggests that the contents of the ulcers may have a hydrogen ion concentration more acid than p_H 7·0.

A consideration of these papers suggests that in any comparisons of amoebicides with emetine *in vitro* the effect of acidity should be studied, particularly when the amoebicides are to be administered orally, and that tests should be carried out at a p_H value of 6·2 or 6·3.

We have carried out a number of tests to study the efficiency of emetine at various hydrogen ion concentrations. In the course of this work we noted that the amount of rice starch added to the tubes of culture medium might also considerably influence the results. Table II summarises the results of three tests,

Table II.

Dilutions of emetine hydrochloride	Test A Medium without 0·2 % phosphate R.S. 0·02 g. p_H 7·7	Test A Medium without 0·2 % phosphate R.S. 0·08 g. p_H 7·57	Test B Medium without 0·2 % phosphate R.S. 0·02 g. p_H 7·15	Test B Medium without 0·2 % phosphate R.S. 0·02 g. p_H 6·2 about	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·65	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·22	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·12
1–1,000,000	O	AA	O	AA	AA	.	AA
1–750,000	.	.	.	.	AA	AA	AA
1–600,000	O	A	O	AA	.	.	.
1–500,000	.	.	.	.	AA (6·57)	AA	AA
1–400,000	O	O	.	AA	.	.	.
1–300,000	.	.	.	AA	O (6·89)	AA (6·53)	AA
1–200,000	O	O	.	AA (5·57)	O (6·57)	O (6·60)	AA (5·97)
1–100,000	.	.	.	AA (5·83)	O (6·46)	O (6·73)	AA (6·33)
1–50,000	.	.	.	.	.	O (6·71)	O (6·49)

Medium = Horse-serum + Ringer's solution.
R.S. = Rice starch.
AA = Living amoebae in original culture.
A = Living amoebae in subculture only.
O = No amoebae observed in original culture or in subculture.

two in medium without phosphate showing the effect of increased acidity and of increased rice starch, and one in medium with buffer showing the effect of increased acidity. Approximately equal amounts of rice starch were added to each tube in a series by preparing a suspension of sterile starch in sterile Ringer's solution and adding equal volumes of this aseptically. The p_H value of this culture medium varies with the methods of determination. The figures quoted were obtained by Mr E. A. Harvey, using the potentiometer and the quinhydrone electrode, those at the top of each column refer to the unsown medium and those inserted in the column to the sown medium after incubation.

If we now proceeded to compare our peak compound, *O-n*-nonylharmol, with emetine under those conditions of acidity which it is suggested may represent conditions *in vivo* more nearly, the ratio of efficiencies showed a considerable alteration (Table III).

Table III.

Culture medium	Starch (per tube) g.	p_H before sowing	p_H after incubation	Lethal concentration *O-n*-Nonylharmol hydrochloride	Lethal concentration Emetine hydrochloride
With phosphate 0·2 %	0·02	6·18	6·2	1–300,000	1–400,000
,,	0·02	6·65	6·46 to 6·89	1–200,000	1–300,000
,,	0·02	6·22	6·53 to 6·73	1–100,000	1–200,000
,,	0·02	6·12	5·97 to 6·49	1–100,000	1–50,000
No phosphate	0·02	6·5	—	1–200,000	1–100,000
,,	0·08	7·4	—	1–100,000	1–100,000

After incubation the hydrogen ion concentration of several tubes was determined, and the limits between which these tubes fell are given in column four. Similar results are obtained if unbuffered medium be used.

It has thus been demonstrated that the amoebicidal value of *O-n*-nonylharmol hydrochloride, although considerably less than that of emetine under optimum conditions for this compound, may nevertheless equal, or even surpass, that of the latter if these conditions are varied slightly, particularly if the reaction of the medium is allowed to become more acid. It has also been pointed out that the literature indicates the possibility that the hydrogen ion concentration in the gut may be in the neighbourhood of p_H 6·2 in cases of amoebic dysentery. It therefore appeared desirable that clinical comparison of *O-n*-nonylharmol hydrochloride with emetine should be carried out, and this substance has therefore been submitted to the Therapeutic Trials Committee of the Medical Research Council with this end in view.

It may also be noted that additional confirmation of the amoebicidal value of *O-n*-nonylharmol is given by Gunn [1932]. Experimenting with *Amoeba proteus*, he found it lethal in 24 hours at a dilution of 1 in 1,000,000 or even 1 in 2,000,000.

SUMMARY.

1. The members of a long series of *O*-alkyl derivatives of harmol have been tested for amoebicidal efficiency *in vitro* in comparison with emetine by the method of Laidlaw, Dobell and Bishop.

2. The most efficient derivative, *O-n*-nonylharmol, has been compared with emetine under different conditions. The results indicate that *O-n*-nonylharmol may be equal to, or even better than, emetine as an amoebicide under appropriate conditions.

I have great pleasure in thanking Mr C. Dobell for his advice when we were about to commence this work and for providing strains of amoebae, Dr F. L. Pyman for general direction of the investigation, Dr T. A. Henry for supplying the *O*-methylpsychotrine sulphate and conessine acid oxalate, and Miss D. Wainman for much careful work at the bench.

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XXXVI. SEPARATION OF CYSTEINE FROM ASCORBIC ACID BY MERCURIC ACETATE.

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(*Received January 5th, 1934.*)

Different investigators have demonstrated that the reducing capacity of plant juices, determined by means of 2 : 6-dichlorophenolindophenol solution, accounts accurately for its observed antiscorbutic potency [Tillmans *et al.*, 1928; 1932; Birch *et al.*, 1933; Harris and Ray, 1933]. This agreement also has been demonstrated with animal tissues [Harris and Ray, 1932; 1933; Birch *et al.*, 1933]. However, biological tests on Jensen rat sarcoma, carried out by Harris [1933], indicate that other reducing substances must be present, because the antiscorbutic activity is much less than would be calculated from the reduction value.

When the titration is carried out in acid medium as indicated by Svirbely and Szent-Györgyi [1933], Birch *et al.* [1933] and Wolff *et al.* [1933], glutathione does not interfere. The presence of cysteine however will be a source of error in the titration with the indicator, because cysteine also reduces the indicator in acid solution [Birch *et al.*, 1933], though not as rapidly as does ascorbic acid. The quantity of cysteine can be determined [Birch *et al.*, 1933] by means of Sullivan's test [Sullivan, 1929, 1, 2], whereas the reduction of iodine solution in acid medium is a measure of the total amount of reducing substances which are present; but we cannot assume that the difference between the total iodine reduction and the cysteine iodine value (calculated from Sullivan's reaction) is due to ascorbic acid. The indicator solution (0·02 % in water) is added from a burette to the solution to be tested (acidified by dilute trichloroacetic acid) till a permanent faint pink colour is obtained. The titration is finished within a minute, whereas the colour generally does not fade after about half a minute. Sometimes this end-point is not sharp, owing to the presence of slowly-reducing substances, because pure ascorbic acid solutions do not show this tendency. Titrating a solution of cysteine-HCl in this way (containing 4 mg. per ml.), 1 ml. solution (acidified by 2 drops trichloroacetic acid) decolorises about 0·4–0·5 ml. indicator after 1 minute. When however 2 ml. indicator solution at once are added to 1 ml. cysteine solution, decoloration takes place in about half a minute. This different behaviour is mentioned because the titration has also been carried out by others by adding the unknown solution to a definite volume of the indicator [Birch *et al.*, 1933]. The two different methods of titration are without influence upon the amount of indicator used by ascorbic acid solutions.

In a previous communication [Van Eekelen *et al.*, 1933] it was stated that cysteine is precipitated by mercuric acetate, whereas ascorbic acid is reversibly oxidised and can be quantitatively regenerated by hydrogen sulphide [Tillmans, Hirsch and Dick, 1932; confirmed by Johnson, 1933].

The ascorbic acid content of solutions of pure ascorbic acid, lemon juice and orange juice was quantitatively recovered by the mercuric acetate precipitation. The reduction with hydrogen sulphide must be effected in a slightly acid

medium, because otherwise, and especially in alkaline solution, reducing sulphur compounds can be formed. It is also important to mention that mercuric acetate solution removes many impurities, a fact which has proved to be very useful in our spectrographic investigations.

Experimental.

Mercuric acetate solution. A 20 % solution in water was prepared and filtered after one day (some hydrolysis takes place).

Precipitation with mercuric acetate solution. To the solution to be tested (which must be slightly acid, excess of acid being removed by $CaCO_3$ and filtration) the mercuric acetate solution is added drop by drop till the precipitation is finished, the reaction taking place in a graduated centrifuge-tube. Care must be taken to avoid a large excess of mercuric acetate, which dissolves the precipitate more or less. After centrifuging, the solution is treated with H_2S. After filtration the solution is left standing overnight, the H_2S removed by nitrogen (controlled by lead acetate paper) and the titration is then carried out.

Example. To 20 ml. of a dilute orange juice solution (titration value 0·5 ml. indicator per ml. solution) 80 mg. crystalline cysteine-HCl were added (4 mg. per ml.). This solution of ascorbic acid and cysteine decolorises after 1 minute about 0·9 ml. indicator per ml. solution (adding the indicator drop by drop to the solution). 18 ml. from this solution (after $CaCO_3$ treatment) required 3 ml. mercuric acetate solution and the procedure given above was followed.

1 ml. solution required 0·43 ml. indicator, calculated $\frac{18}{21} \times 0{\cdot}5 = 0{\cdot}43$ ml. The sodium nitroprusside test was completely negative.

Summary.

Cysteine, being a source of error in the determination of ascorbic acid by means of 2 : 6-dichlorophenolindophenol solution, is removed quantitatively by mercuric acetate solution.

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Note added February 6th, 1934. Ergothioneine, which reduces the indicator in acid solution, is also precipitated by mercuric acetate in acid medium.

XXXVII. THE EFFECT OF GUANIDINE DERIVATIVES ON TISSUE RESPIRATION.

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(*Received November 23rd, 1933.*)

The full review of the problem of tetania parathyreopriva recently published by Thomson and Collip [1932] makes it clear that many points in connection with the biochemical mechanism of the production of tetany remain open to question. Whilst it is universally agreed that the outstanding chemical change to be observed in tetania parathyreopriva is the lowering of the blood-calcium, it is still maintained by some authors that this phenomenon is incidental and is only one result of the general intoxication with guanidine derivatives which they regard as the primary cause of the condition.

There is at least no doubt that guanidine derivatives do produce symptoms of tetany, when administered in amounts so small as to indicate that the effect is a genuine intoxication and is not referable to any gross change such as a shift in the acid-base balance of the blood due to the basic character of the guanidine.

It is evident that any information as to the actual physiological effect of guanidine may be expected to be of use in elucidating the mechanism of the production of tetany by this substance and in helping to solve the further question as to whether intoxication with guanidine has in fact anything to do with tetania parathyreopriva.

The object of the present note is to describe the effect of guanidine derivatives on the respiration of isolated tissues.

Experimental.

The statement of Meyerhof [1921] that guanidine depresses the rate of yeast fermentation was confirmed.

For our own investigation we have used for the most part the brains of pigeons and rats; in addition experiments have been made with rat's liver and muscle and with rabbit's blood and nerve. The guanidine derivatives employed were guanidine hydrochloride and carbonate and methylguanidine hydrochloride.

The oxygen consumption of the tissues in air was measured in Dixon and Elliott's [1930] modification of the Barcroft apparatus, the details of the method being the same as those described in a former paper [Gavrilescu and Peters, 1931] except for the preparation of the material. It has been found that in experiments of this type the fineness and uniformity of division of the tissue are of the greatest importance if concordant results are to be obtained; discrepancies previously observed [*cf.* Gavrilescu and Peters, 1931] are probably to be ascribed to insufficient attention to this point. In the present series the liver, removed immediately after the death of the animal, was forced with a bone spatula through a fine sieve; the muscles were crushed in a Latapie grinder. In this way

the tissues were obtained in a finely divided and homogeneous condition, and duplicate determinations usually showed differences of less than 2 %.

0·1 g. of tissue was suspended in 3 ml. Ringer solution in the shaking-bottle of the Barcroft apparatus; in the case of experiments with guanidine derivatives the latter were added, immediately before placing the apparatus in the thermostat, in the form of 0·1 ml. of a 1·5 % solution; the final concentration of guanidine derivative was therefore 0·05 %. In a few experiments (see Table I) this concentration was increased. No change in p_H resulted from the additions of guanidine derivatives. The results are shown in Table I.

Table I.

Average oxygen uptake in mm.³/g./hr.

	Control	Guanidine	Para-thyrine	Guanidine and para-thyrine	% lowering by guanidine
A. Pigeon's brain	1428	1291			9·6
	1497	1399			6·5
	1516	1468			3·1
	1638	1444	1564		11·9
	1521	1258	1464		17·3
	1336	1239	1286		7·1
	1667	1576 (1592, 1341)*			5·4 (5·0, 19·6)
	1344	1329 (1252, 1053)*			1·2 (6·8, 21·7)
B. Rat's brain	1723	1515	1727	1558	12
	1375	1291	1403		6·1
	1744	1347	1629		22·7
	1610	1476	1621		8·3
	1560	1347	1741	1503	7·2
	1777	1567	1604		11·9
	1476	1287			12·9
	1734	1377†	1604	1420†	20·6
	1763	1427†	1747	1418†	19
	1728	1435†	1687		17
C. Rat's liver	572	536	544	540	6·3
	655	611			6·7
	706	692			—
	755	722			4·4
	974	1076			—
	572	573			—
	570	542			5·0
D. Rat's muscle	703	611			13
	175	222			—
	244	268			—
	253	215			15
	286	215			25
E. Rabbit's blood	149	156	191		—
	245	224	222	229	8·6
	222	207	234	182	7·1
	226	219			3·2
	200	191			4·5
	316	265			16
	349	311			10
F. Rabbit's sciatic nerve	277	207			25

* Guanidine concentrations 0·25, 0·5, 1·2 %.
† Methylguanidine.

In Table II are seen the results of an attempt to perform a similar experiment *in vivo*. Col. 1 shows the oxygen uptakes of the brains of six normal rats and col. 2 those of the brains of three rats killed at the height of the symptoms of tetany induced by intraperitoneal injection of 0·5 g./kg. of guanidine.

Table II.

Oxygen uptake of rat's brains in mm.³/g./hr.

	Normal brains	Brains of animals killed at height of tetany after intraperitoneal injection of 0·5 g./kg. guanidine or methylguanidine
	1723	1283
	1744	1511
	1610	1594
	1734	—
	1763	—
	1728	—
Average	1717	1462

Discussion.

It is clear from Tables I and II that in almost all cases the presence of guanidine derivatives has a significantly depressant action on the oxygen uptake of the tissues examined. It may be noted that the effect is less marked with pigeon's brain than with rat's brain, and this may be correlated with the known fact that the toxic dose of guanidine is about twice as high for pigeons as for rats.

It seems evident therefore that the general toxic effect of guanidine, leading to the production of symptoms of tetany, is to be ascribed in part at least to depression of tissue metabolism, in particular the metabolism of the brain. It is possible that this depression may lead to increased irritability of the respiratory centre and hence to alkalosis owing to excessive pulmonary ventilation.

The few experiments in which parathyrine was added gave no indication of a direct antagonistic action between the parathyroid hormone and the guanidine derivatives; our work does not therefore so far throw any light on the possible connection of guanidine intoxication with tetania parathyreopriva.

Summary.

The oxygen uptake of isolated tissues (brain, liver, muscle) is significantly depressed by the presence of guanidine or methylguanidine in approximately 0·05 % concentration.

The oxygen uptake of the brain of rats poisoned with guanidine is lower than that of normal rat's brain.

We are indebted to Prof. I. Cantacuzino for help with the animal experiments.

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XXXVIII. THE DETERMINATION OF AMINO-ACIDS IN WHEAT FLOUR.

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(*Received December 6th, 1933.*)

THE work here reported was undertaken as a preliminary to the investigation of the changes in the amino-acid content of flour dough during fermentation. In order to study these changes it is necessary to stop the enzyme action at different times before extracting and estimating the amino-acid content, unless the extract can be prepared in a time negligibly short in comparison with the time that fermentation has proceeded, and provided that there is no production of amino-acid in the aqueous extract. A disadvantage of the freezing method employed by Brownlee and Bailey [1930] to inhibit enzyme action is that the enzymes are not destroyed, and may become active during the extraction if this is carried out at normal temperatures, so that observed results may not necessarily be due to fermentation in the dough.

Denham and Scott Blair [1927] found that the variations in amino-acid content of flour-water suspensions for the first few hours after mixing, which were described by Swanson and Tague [1916; 1917], also occurred in the clear extract of the flour and concluded that the proteolytic activity of a flour could be measured by the increase in amino-acid content of the extract. The method of estimation used by Denham and Scott Blair, however, is open to criticism, as shown later. Swanson and Tague [1916] found that the amino-acid content of a flour extract increased on standing, but as their estimations were made weekly they are not comparable with those of Denham and Scott Blair [1927]. Herd [1931] reported that "the change in amino-nitrogen content of the flour extracts is negligible in all cases over a period of 118 hours," but the change in titre reported by Denham and Scott Blair [1927] would not cause a significant change in the titres reported by Herd [1931], and differences in technique render the two sets of results not strictly comparable.

In view of these apparently conflicting statements it seemed advisable to investigate the change in amino-acid content of a flour extract on standing, particularly for the period immediately after the separation of the flour.

Methods based on the formaldehyde titration of Sørensen [1907, 1] and the alkalimetric titration of Foreman [1920] have been used in flour work, and the Sørensen method has always been found the most suitable, though in the data published by Cairns and Bailey [1928] on a comparison of the two methods, the Foreman titration was carried out in 50 % alcohol, and its rejection was therefore not entirely justified since in 50 % alcohol the polypeptides would titrate but only a part of the amino-acids [see Willstätter and Waldschmidt-Leitz, 1921].

EXPERIMENTAL.

Although the procedures described by Swanson and Tague [1916] (for the Sørensen titration), by Foreman [1920] and by Harris [1923] (for a modified Foreman titration) all gave results accurate to within a few per cent. of the theoretical figures with pure amino-acids, these methods were

found to be unsuitable for flour extracts, the Foreman and Harris methods because they involve the dilution of the extract with nine volumes of 95 % alcohol (and the amino-acid content of the extract is originally very small) and the Swanson and Tague method because the slight opalescence of the flour extract renders the final end-point with phenolphthalein or thymolphthalein somewhat indefinite. The technique recommended by Brown [1923] for the formaldehyde titration of bacteriological media was therefore tried and was found very satisfactory.

To 10 ml. of flour extract was added 0·2 ml. of phenol red solution and the solution titrated to p_H 8·0 with $N/14$ NaOH, using a micro-burette calibrated to 0·01 ml. The end-point was obtained by matching with a buffer solution of p_H 8·0 in a comparator block. This titration (stage 1) represents the titratable acidity of the extract. To the sample were then added 8 ml. of commercial 40 % formaldehyde, 0·18 ml. of indicator solution, and the mixture was again titrated to p_H 8·0. (0·18 ml. of indicator was added because the blank titration on the formaldehyde is about 1 ml.). This second stage titration represents the alkali required both to neutralise the amino-acid in solution and to bring the formaldehyde to p_H 8·0. In each case suitable screens of extract and of diluted extract were used in the buffer side of the comparator block. This second stage titration was corrected by subtracting from the titre a blank titration to p_H 8·0 of 8 ml. of formaldehyde diluted with 10 ml. of distilled water instead of bringing the formaldehyde to p_H 8·0 before adding to the flour extract. No change in the blank titration was ever observed during the few hours occupied by the experiments to be described.

The end-points obtained by this method were very sharp and distinct and were sensitive to less than 0·01 ml. of $N/14$ NaOH. An endeavour to increase the sensitivity of the method by using $N/100$ alkali was not very successful owing to lack of distinctness of the end-point. Other indicators offered no advantage.

It is recognised that, under the conditions of this work, titrating the test solution to a colour match with the standard buffer solution does not necessarily mean that the p_H of the test solution and the buffer solution are the same [see Halton and Fisher, 1928], but the conditions are at least reproducible. Moreover, the electrometric determination of the p_H of a solution containing 16 % of formaldehyde would introduce difficulties in the error of the junction potential with the formaldehyde solution.

Table I. *Amino-nitrogen in flour extract at various times.*

Titration	Time (mins.)	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (*a*)			
1	0	0·21	0·30
2	10	0·21	0·30
3	20	0·21	0·30
4	60	0·22	0·30
5	90	0·22	0·29
6	120	0·21	0·30
7	165	0·21	0·31
8	225	0·21	0·30
9	345	0·22	0·30
10	495	0·21	0·31
Flour (*b*)			
1	0	0·24	0·30
2	10	0·24	0·30
3	25	0·23	0·31
4	40	0·22	0·30
5	75	0·23	0·30
6	120	0·22	0·30
Flour (*c*)			
1	0	0·31	0·41
2	20	0·32	0·42
3	40	0·32	0·41
4	70	0·32	0·41
5	120	0·33	0·41
6	180	0·32	0·41

For the preparation of the extract the method described by Denham and Scott Blair was used. Flour, equivalent to 40 g. dry flour, was extracted by shaking for five minutes with 200 ml. of distilled water to which toluene had been added, the mixture centrifuged and the slightly opalescent extract decanted through a filter. The filtrate was titrated immediately and after various intervals of time. Representative results, given in Table I, for three different flours, (*a*) from a typical mill mixture, (*b*) from English wheat, (*c*) a 30 % low grade flour, show that there was no change in the amino-acid content of the extract on standing in the presence of toluene, nor was there any change in titratable acidity.

Further experiments in the absence of toluene gave the same results, thus confirming the finding of Denham and Scott Blair that for short period experiments toluene did not affect the results. Similar results were obtained when the accuracy of the method was increased five-fold by titrating 50 ml. of solution instead of 10 ml., so that the failure to observe an increase in amino-acid content with time was not due to the insensitivity of the method. Experiments were conducted in which the extract was maintained at 25° and 37° respectively, but no increase in amino-acid titration was obtained.

That the flours used exhibited the phenomenon of increase in amino-acid content with increasing time of extraction is shown in Table II. This table also shows the increase in titratable acidity (stage 1) with increasing time of extraction. This increase is due, at least in part, to an increase in the buffer value of the extract, caused by the production of inorganic phosphate from phytin by the action of phytase [Bailey and Peterson, 1921], but may also be due to the production of organic acids by fermentation processes. The low-grade flour (*c*) shows a much greater increase in amino-nitrogen than flours (*a*) or (*b*).

Table II. *Increase in amino-nitrogen with time of extraction.*

Time of extraction	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (*a*)		
5	0·19	0·30
20	0·27	0·32
35	0·32	0·35
50	0·33	0·35
Flour (*b*)		
5	0·24	0·30
15	0·28	0·33
30	0·33	0·33
55	0·40	0·37
75	0·44	0·38
105	0·49	0·39
Flour (*c*)		
5	0·31	0·42
30	0·56	0·48
60	0·73	0·54
120	0·85	0·55

Since the results shown in Table I are directly opposed to the findings of Denham and Scott Blair, the work was repeated, using the method of estimation described by these authors (an acid titration of one portion to p_H 5·5 with methyl red and an alkali titration of a second portion to "a full rose colour" with phenolphthalein[1] in the presence of formaldehyde) both in the presence and absence of toluene. With the mill mixture and English flours no increase in amino-acid content was obtained either with or without toluene, but the extract of the low-grade flour showed an increase in the titration with time whether

[1] For this end-point a buffer solution of p_H 9·0 was used in the work here described.

Table III. *Amino-nitrogen content of extract of low-grade flour (no toluene).*

	Denham and Scott Blair method			Brown method	
Time mins.	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour	Stage 1	Stage 2, mg. amino-N per 2 g. flour
0	0·11	0·77	0·88	0·32	0·41
20	0·11	0·85	0·96	0·31	0·41
40	0·11	0·88	0·99	0·31	0·40
60	0·12	0·90	1·02	0·31	0·41
120	0·14	0·91	1·05	0·31	0·41

toluene were present or not. Some typical figures are given in Table III which show an increase in the amino-nitrogen content of the extract as estimated by the method of Denham and Scott Blair and no increase by the technique of Brown. Table IV shows the absence of any change in amino-nitrogen content of the "mill mixture" and the English flour and the increase in the case of the low-grade flour by the Denham and Scott Blair method irrespective of the presence or absence of toluene.

Table IV. *Amino-nitrogen content of flour extracts with and without toluene.*

	Denham and Scott Blair method					
	Without toluene			With toluene		
Time mins.	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour
English flour						
0	0·06	0·63	0·69	0·06	0·63	0·69
30	0·06	0·62	0·68	0·06	0·63	0·69
60	0·06	0·64	0·70	0·06	0·63	0·69
120	0·06	0·63	0·69	0·06	0·63	0·69
240	0·06	0·63	0·69	0·06	0·63	0·69
Mill mixture						
0	0·07	0·60	0·67	0·07	0·61	0·68
30	0·07	0·60	0·67	0·07	0·60	0·67
60	0·07	0·61	0·68	0·07	0·61	0·68
120	0·07	0·61	0·68	0·07	0·61	0·68
Low grade flour						
0	0·10	0·79	0·89	0·11	0·80	0·91
30	0·11	0·88	0·99	0·11	0·89	1·00
60	0·11	0·92	1·03	0·12	0·92	1·04
120	0·12	0·94	1·06	0·13	0·94	1·07
180	0·13	0·96	1·09	0·13	0·95	1·08

The slight discrepancies between the amino-nitrogen content of the low grade extract with and without toluene are due to the technique of this experiment which was to prepare several hundred ml. of extract, divide this into two portions and add toluene to one portion. Each portion was then titrated in turn, so that before the initial titration the "toluene" extract had stood a slightly longer period than had the "no toluene" extract, and this is reflected in the slightly higher amino-nitrogen content of the "toluene" extract.

Table III also illustrates the great difference in the absolute magnitude of the amino-acid content as determined by the two methods; that obtained by the Denham and Scott Blair method (the sum of the two titrations) being some two to three times as great as that obtained by titrating to the same p_H before and after the addition of the formalin solution. This discrepancy is due to the titratable acidity and buffer capacity of the flour extract which are included as amino-acid by the Denham and Scott Blair method.

Though the main object of this investigation had been accomplished in showing that no measurable proteolysis occurred in the flour extracts for the few hours immediately subsequent to the separation of the flour, it was thought to be of interest to try and explain the anomalous result obtained with the low-grade flour. Extracts of this flour, prepared as described above, were analysed in the following manner after various periods of standing. 10 ml. of extract were titrated with $N/14$ HCl to p_H 5·5 as in the Denham and Scott Blair method. To another 10 ml. was added 0·2 ml. of thymol blue indicator and the solution titrated to match successively the colours in buffers of p_H 8·0, 8·5, 8·7 and 9·0; formaldehyde and further indicator solution were then added and the mixture again titrated to these end-points. Table V shows the results obtained in one such experiment when toluene water was used for the extraction. The amino-acid by the method of Denham and Scott Blair is the sum of the titration to p_H 5·5, the titratable acidity and the amino-acid by the method of Brown.

From Table V it is seen that there is no change in the buffer value of the solution as shown by the acid titration to p_H 5·5 and the alkali titrations to

Table V. *Determination of amino-acid at various times in the aqueous extract of a low-grade flour by the methods of Brown and of Denham and Scott Blair.*

Time in mins.	from first titration		0	20	40	60	120
	from mixing		20	40	60	80	140
Acid titration to p_H 5·5; ml. $N/14$ HCl			0·10	0·10	0·10	0·10	0·10
p_H 8·0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·31	0·31	0·31	0·31	0·31
	Amino-nitrogen mg. per 2 g. flour	Brown	0·42	0·41	0·42	0·42	0·41
		Denham and Scott Blair	0·83	0·82	0·83	0·83	0·82
p_H 8·5	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·39	0·38	0·39	0·39	0·38
	Amino-nitrogen mg. per 2 g. flour	Brown	0·38	0·38	0·38	0·38	0·38
		Denham and Scott Blair	0·87	0·86	0·87	0·87	0·86
p_H 8·7	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·41	0·40	0·41	0·42	0·41
	Amino-nitrogen mg. per 2 g. flour	Brown	0·36	0·35	0·35	0·34	0·35
		Denham and Scott Blair	0·87	0·85	0·86	0·86	0·86
p_H 9·0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·48	0·48	0·48	0·48	0·48
	Amino-nitrogen mg. per 2 g. flour	Brown	0·28	0·33	0·38	0·40	0·48
		Denham and Scott Blair	0·86	0·91	0·96	0·99	1·06

p_H 8·0, 8·5, 8·7 or 9·0 respectively. Also this latter titration indicates that there is no change in the titratable acidity of the extract on standing. The amino-acid by the method of Brown is less the higher the p_H to which the solution is titrated, in accordance with the theory of the titration. At p_H 8·0, 8·5 and 8·7 there is no increase in amino-acid as estimated by either method, but at p_H 9·0 there is an increase by both methods.

It was suspected that this increase at p_H 9·0 was due to the production by enzyme action of substances containing both carboxyl and amino-groups but which did not become titratable even under the action of formaldehyde until p_H 9·0 was reached. If so, such increase might be enhanced were the enzyme

allowed to act on the flour substrate instead of on extracted protein only. A suspension of 40 g. of "dry" flour in 200 ml. of distilled water was allowed to autolyse at 27°. From time to time samples were taken, centrifuged, filtered and the filtrate titrated to p_H 8·0, 8·5, 8·7 and 9·0 respectively as before. The results of one such experiment are shown graphically in Fig. 1, plotting amino-nitrogen against time.

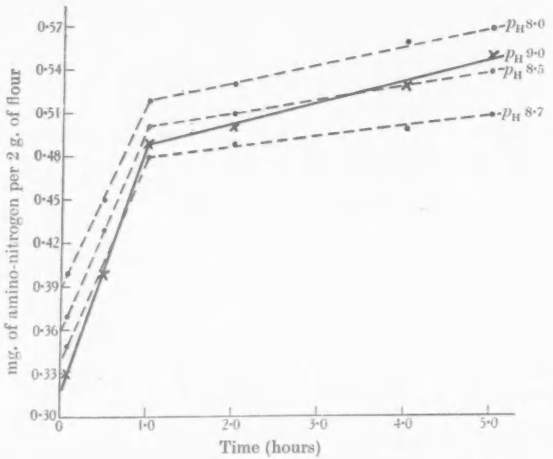

Fig. 1. Amino-nitrogen in flour-water digest at various times.

Initially the amino-acid content is lower the higher the p_H used, as is to be expected, and all values increase with time. This increase is due to further extraction[1] of the amino-acids from the flour and also to the production of amino-acids from the proteins. The rapid increase for about an hour is due almost entirely to extraction, the subsequent slow increase is due to proteolysis, and the slope of this second portion of the graph measures the proteolytic activity of the flour. The graphs at p_H 8·0, 8·5 and 8·7 are practically parallel, but the graph for p_H 9·0 crosses the other graphs, indicating the more rapid production of substances titratable at p_H 9·0.

The addition of yeast to a flour-water suspension did not enhance this effect, because the amino-acid produced was used by the yeast in its metabolism, and this rendered difficult the interpretation of the results from the standpoint of the present work. The indications were, however, that at each estimation after the first a greater titration was obtained at p_H 9·0 than at p_H 8·0 after the addition of formaldehyde. There was also evidence that the titration at p_H 8·7 was increased in the presence of yeast.

Table VI shows the results obtained from a takadiastase digest of the gluten washed from this flour. The amino-nitrogen estimated at p_H 9·0 increases more

[1] It may be noted that extraction was complete in one hour, so that the method of estimating amino-acids in flour given in the Methods of Analysis of the Association of Official Agricultural Chemists, p. 168 (3rd edition, 1930), which requires extraction for a three-hour period, measures not only the amino-acid originally present, but also that produced by some two hours' proteolysis, unless the extraction be carried out at a low temperature.

Table VI. *Amino-nitrogen (mg. per 10 ml. of solution) formed in a gluten-takadiastase digest.*

Time hours	Titration	p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
0	1	0·06	0·06	<0·07	<0·08
	2	0·00	0·00	0·00	0·00
1	1	0·10	0·12	0·13	0·14
	2	0·05	0·03	0·02	0·02
2	1	0·12	0·14	0·15	0·16
	2	0·06	0·04	0·06	0·07
3	1	0·14	0·16	0·17	0·18
	2	0·09	0·07	0·09	0·10
4	1	0·16	0·18	0·19	0·21
	2	0·12	0·10	0·12	0·13
5	1	0·18	0·20	0·22	0·23
	2	0·14	0·12	0·14	0·18

Titration 1: initial alkali titration.
Titration 2: alkali titration in presence of formaldehyde less the formaldehyde blank, *i.e.* amino-nitrogen.

rapidly than that at p_H 8·0. In all cases the amino-nitrogen at p_H 8·5 is less than that at p_H 8·0 for the corresponding time in accordance with theory, but it is clear that the substances affecting the estimation at p_H 9·0 are also exercising an influence at p_H 8·7, but to a lesser extent. The action of takadiastase on the gluten is thus comparable with the changes occurring in the flour extract and in the flour suspension.

No such abnormal increase at p_H 9·0 was obtained in pepsin digests of gluten, caseinogen or egg-albumin.

An acid hydrolysis of the moist gluten from this low-grade flour was followed by estimation of the amino-acid from time to time by titration to p_H 8·0, 8·5, 8·7 and 9·0 respectively as before. The results shown in Table VII indicate that hydrolysis was normal, and that the amino-acid estimated at p_H 9·0 is always less than that at p_H 8·0.

Table VII. *Amino-acid formed in acid hydrolysis of flour-gluten.*

Time hours	mg. of amino-nitrogen at p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
0	0·00	0·00	0·00	0·00
0·5	0·65	0·62	0·60	0·52
1·0	0·84	0·81	0·80	0·76
2·0	0·93	0·88	0·88	0·82
3·0	1·02	0·98	0·98	0·91
6·0	1·06	1·03	1·03	0·98
9·0	1·09	1·05	1·05	0·98

To determine, if possible, the class of substance responsible for the abnormal increase, various materials probably occurring in the flour-water suspension were titrated to p_H 8·0, 8·5, 8·7 and 9·0 respectively before and after the addition of formalin. Neither protein, peptone nor amino-acid solutions showed any increased titration at p_H 9·0. Indeed all showed the expected decrease in titration with increase in p_H value. These results are shown in Table VIII. All possible mixtures of these solutions were titrated similarly, and the values obtained were strictly additive and gave no evidence of an increased titration

at p_H 9·0 but showed the same reduction in titration as is illustrated in Table VIII. In each case the requisite amount of solution was taken, diluted to 10 ml. and then titrated.

Table VIII. *Amino-acid estimation at* p_H *8·0, 8·5, 8·7 and 9·0 respectively.*

		Phenol red		Thymol blue			
Titration ml. *N*/14 NaOH		p_H 7·0	p_H 8·0	p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
Egg-albumin	1	0·03	0·08	0·08	0·09	0·10	0·11
	2	0·21	0·25	0·25	0·24	0·23	0·20
Peptone	1	0·06	0·11	0·11	0·16	0·18	0·20
	2	0·18	0·18	0·18	0·15	0·14	0·09
Alanine (theory 0·91 ml.)	1	0·01	0·02	0·02	0·06	0·09	0·12
	2	0·74	0·89	0·89	0·87	0·86	0·81
Glutamic acid hydrochloride (theory 0·91 ml.)	1	1·82	1·83	1·83	1·89	1·92	2·01
	2	0·57	0·88	0·88	0·83	0·81	0·76

Titration 1: solution without formaldehyde.
Titration 2: difference between titrations before and after the addition of formaldehyde corrected for the blank titration, *i.e.* titration due to amino-acid.
Owing to the large titration to neutralise the solution of glutamic acid hydrochloride, extra indicator was added to obtain the same indicator concentration as in the buffer solution. Also the formaldehyde blank was determined on 8 ml. of formalin diluted with 12 ml. of distilled water.

Discussion.

The results obtained in this study of the estimation of amino-nitrogen in wheat flour show that extraction is complete in about one hour, and that no further formation of amino-acid occurs in the extract after separation from the flour. In the case of one flour, a low-grade, there was an apparent increase in amino-nitrogen in the extract when the estimation was carried out at p_H 9·0, but no increase at the lower p_H values used.

This increase is not due to more complete neutralisation of the amino-acids at the higher p_H, for the more recently published titration curves for individual amino-acids in the presence of formaldehyde show that neutralisation is complete at p_H 8·0[1] [Brown, 1923; Harris, 1929]. In Table VIII the low results at p_H 7·0 are due to incomplete neutralisation of the amino-acid in the presence of formaldehyde, and the low results at $p_H > 8·0$ are due to partial neutralisation of the amino-acids before the addition of formaldehyde. That the maximum titration at p_H 8·0 is slightly less than the theoretical titration value of 0·91 ml. is also due to this latter factor.

Moreover, the facts that in the case of the flour extract, the flour suspension and the takadiastase-gluten digest, lower titrations were recorded at p_H 8·5 than at p_H 8·0, and that there is not a progressive increase in titration with increase in p_H indicate that completion of neutralisation of the amino-acids is not the explanation of the phenomenon.

If it be accepted that the formaldehyde titration estimates carboxyl groups that become titratable because of the action of formaldehyde on the amino-groups, then the observed increase at p_H 9·0 is due to an increase in the number

[1] Since the above was written a recent paper by Van Slyke and Kirk [1933] has been received which shows titration curves of amino-acids compiled from various authors. The titration curves in the presence of formaldehyde show that neutralisation is not complete before p_H 9·0. However, these curves were "estimated from end-points at p_H 9 to 10, given by Sørensen" [1907, 2], and are said to be "only approximately exact" [Van Slyke and Kirk, 1933, p. 657], so that the experimental curves of Brown [1923] and of Harris [1929] are the more reliable.

of carboxyl groups (or amino-groups affected by formaldehyde) which, even in the presence of formaldehyde, do not titrate until p_H 9·0 is reached. That such increase is not due to the production of amino-acids in the usual sense of this term is shown by the titration values at p_H 8·0. Nor is this increase due to the production of proteoses or peptones, as shown by a pepsin digestion, though it occurs in a takadiastase digest of gluten. It is therefore due to substances formed in the enzymic hydrolysis of the proteoses or peptones formed from gluten. A combination of carboxyl and amino-groups in a compound of very low acid dissociation constant may be expected in a substance intermediate between the polypeptides and the amino-acids. Such a substance, under the action of formaldehyde, would not affect the amino-acid estimation at p_H 8·0 because of its very low acid dissociation constant but would become titratable at p_H 9·0. Then in the flour extract, if the proteins present are broken down, not to their constituent amino-acids but to some slightly larger grouping of a few amino-acids, this would be estimated at p_H 9·0 and not at p_H 8·0 and would explain the experimental results.

Although only one of the four flours examined to date has shown this phenomenon of increase in amino-nitrogen at p_H 9·0 and not at p_H 8·0, it seems from the work of Denham and Scott Blair and of Halton (unpublished) that it may be of more common occurrence than this would indicate.

In considering the question of protein changes in a flour dough it is necessary to take into account any such change as is indicated in this paper, for the physical properties of the dough will be affected by any protein degradation whether the decomposition be carried as far as the amino-acid stage or not.

Perhaps it should be emphasised that titration to p_H 9·0 before and after the addition of formaldehyde is not advocated as a method of estimating amino-acids, but that in conjunction with an estimation at p_H 8·0 it may yield some information not afforded by the amino-acid estimation alone.

SUMMARY.

1. The claim that proteolysis occurs in flour extracts has been studied for the few hours immediately subsequent to separation of the extract.

2. The technique of Brown for the estimation of amino-acids by the Sørensen method has been applied to flour extracts.

3. Proteolysis, as measured by the production of amino-acids, did not occur in the flour extracts studied.

4. For one flour of the four studied (a low-grade) the amino-acid determinations made on the extract at varying p_H values showed an increase with time if the solution were titrated to p_H 9·0 before and after the addition of formaldehyde, but no increase if the titration were carried to p_H 8·0 only.

5. It is suggested that this is due to the enzymic production of substances containing carboxyl and amino-groups and having a low acid dissociation constant, which do not titrate even in the presence of formaldehyde until p_H 9·0 is reached.

The author is indebted to the University of Western Australia for the award of a Hackett Research Studentship during the tenure of which this work was carried out, to Sir John Russell, Director of Rothamsted Experimental Station, for placing at his disposal the facilities of the Station, and to the Research Association of British Flour Millers for the flours used in this work. His thanks are due to Dr A. G. Norman, Dr P. Halton and Mr G. W. Scott Blair for their assistance and advice.

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XXXIX. A HORIZONTAL MICRO-BURETTE.

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(*Received December 22nd, 1933.*)

In titrations where we are restricted to determining minute quantities it is necessary to keep the final volume of fluid as low as possible, for in this way we decrease the amount of added reagent necessary to produce an observable change in concentration around the end-point and so minimise the total error. The accurate delivery of small volumes needs special burettes. With the Bang 2 ml. burette the percentage error of delivering 2 ml. is greater than the percentage error of delivering 50 ml. with the ordinary burette. Expressed as a coefficient of variation the ratio is in fact 0·11 to 0·05. Owing however to the large reduction of volume, and provided we are dealing with quantities below a certain level (about 10^{-5} equivalent), increased accuracy is secured with the micro-burette, the advantage being greater the smaller the quantity. At the same time the use of standard solutions ten times more concentrated—and hence in general more stable—is an important added advantage.

It is occasionally required to titrate quantities so minute that a burette delivering volumes of the order of 0·1 ml. is necessary. If the principle of delivering from a vertical burette is retained the delivery error alone rises to 0·45—expressed as a coefficient of variation. In Rehberg's burette [Rehberg, 1925] this delivery error appears to be much lessened by the principle of expelling the fluid by a mercury column controlled by a screw. The percentage error of delivery however can be reduced to about one-fourth the above figure by the simple expedient of delivering from a horizontally placed graduated tube. The accuracy obtained is as great as in Rehberg's burette but the ease of construction and cleaning is much greater. The percentage error of delivering 0·1 ml. with the horizontal burette here described is practically identical with the error of delivering 1 ml. with the Bang 2 ml. burette. It can therefore be substituted for the Bang burette over the whole range of micro-work. It titrates minute quantities (of the order 10^{-6} equivalent, or less) with substantially greater accuracy and permits the use of fluids ten times more concentrated and under better conditions of security from contamination by the carbon dioxide of the air or by alkali from glass surfaces—the delivered fluid having been in contact only with pyrex or paraffined walls.

Description of the burette.

Fig. 1 illustrates the structure of the burette. *A* is a piece of thermometer tubing of approximately 1 mm. bore graduated in centimetres and millimetres and placed horizontally. It is joined by a small length of pressure tubing to tube *B*. To *B* is attached the delivery tube and tap *C* and also the connecting tube and tap *D* communicating with the small container *E* on a hinged platform with support. Behind tap *D* a small window is cut in the wooden supporting

frame to allow free manipulation. All tubing except the graduated thermometer tubing (and this also if possible) should be made of pyrex glass. An advantage may be secured if the tube adjoining the graduated tube has twice or more the internal bore of this latter for then the volume of fluid in the graduated tube will be small in comparison and the emerging fluid will have been in contact only with the pyrex glass or the paraffined walls of the container. The cleansing required for a smoothly running meniscus is very easily done by detaching the graduated tube and cleaning by any of the ordinary methods. It may as a rule be cleaned sufficiently well using alcohol and ether. After cleansing it is replaced with the glass ends in contact and washed out with a

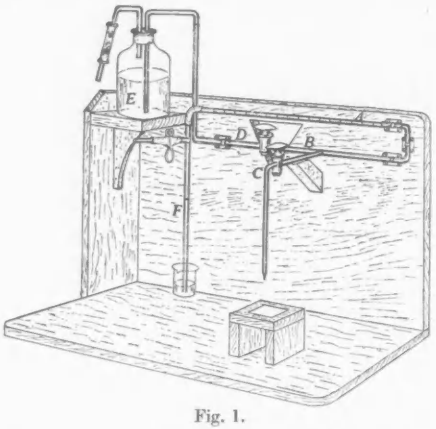

Fig. 1.

few drops of fluid from the container. The small hydrostatic pressure (5, 10 or more cm.) ensures a uniform delivery without drainage owing to the horizontal position. The rate of delivery may be found inconveniently rapid unless the delivery tip is very finely constricted. It is then liable to become blocked by any small solid particle. A simple and efficient method of adequately controlling the full delivery rate with open tap so that fine adjustments of the meniscus may be made on turning consists in attaching a glass tube to the end of the graduated section as in Fig. 1 (*F*). This dips into water below, contained in—say—a 25 ml. beaker. By sucking up some water in this tube and attaching the clip, the delivery pressure of the burette is diminished and controlled as desired.

The use of good quality thermometer tubing will ensure uniformity of bore and lengths of 25 or 50 cm. may be used as desired. With an internal bore of 1 mm. approximately 128 divisions on the burette correspond to a volume of 0·1 ml. For the container a 100 ml. reagent bottle—paraffined inside when weak alkalis are used will be found suitable.

The vertical length of the delivery tube may be varied to suit the particular fluid containers used, whether small beakers, centrifuge-tubes or the special absorbing apparatus described in a previous communication [Conway, 1933]. A standard length of about 10 cm. will suit nearly all containers. Along this delivery tube a fine pyrex tube may be attached by an elastic band or bands at the top and connected with an air-blower for stirring when centrifuge-tubes

or other such containers are used. The tip of the delivery tube may be immersed in the fluid to be titrated or immersed after the emergence of some fluid. If this method is not used a fine pyrex rod will be found suitable for removing small quantities of fluid from the tip and an amount representing a millimetre division on the graduated tube may be quite easily controlled in its emergence and transference to the titrated fluid.

The standardisation of the burette can be carried out by weighing the total of five or more deliveries of the graduated section with subsequent reference to correction tables for transferring grams to millilitres [*e.g.* those given by Stott, 1928]. A titration method may be also used. In the actual use of the burette it is however rarely required to know the volume delivered in ml., the length of the fluid column in the graduated tube corresponding on titration to a measured quantity of standard fluid being taken as the reference figure. This has the advantage of standardising at the same time the fluid in the burette.

To illustrate the action of the burette the variable error of delivering 0·1 ml. will be considered as well as a series of titrations of $10^{-3.7}$ milli-equivalents of sulphuric acid.

The variable error of delivering 0·1 ml. expressed as a coefficient of variation.

This error was investigated by a titration method. Deliveries of 0·1 ml. of N sulphuric acid represented by 128 mm. on the graduated tube were made into a small pyrex dish. The deliveries were successively titrated with $N/10$ carbon dioxide-free alkali from a 2 ml. standard Bang burette, a little indicator mixture of methyl red and methylene blue being added. A similar series of titrations was carried out where 1·0 ml. of $N/10$ sulphuric acid was delivered from another standard 2 ml. burette. The amount of acid titrated in both series was therefore the same and the amount of alkali necessary to change the colour of the indicator of negligible quantity owing to the strength of the solutions. The only difference affecting the variable error between the two sets is that arising from the delivery of 0·1 ml. from the horizontal burette and of 1 ml. from the Bang burette. The difference of the squares of the coefficients of variation for the two sets of titrations is therefore the difference between the squares of the coefficients for delivering these volumes. The coefficients of variation for the two sets were 0·26 and 0·25 respectively. Also the coefficient of variation for delivering 1 ml. from the Bang burette was found from 20 successive weighings to be $0{\cdot}11 \pm 0{\cdot}02$ so that it may be readily calculated that the coefficient for the horizontal burette delivery of 0·1 ml. is 0·12. Subsequent and similar series gave 0·10 and 0·15, so that 0·12 may be taken as the approximate coefficient of variation for delivering 0·1 ml. with the burette described here.

The variable error of titrating 1 ml. of 0·0002 N *sulphuric acid illustrating the minimum variable error of acidimetric titration.*

The object of determining this error is to ascertain the least amount of alkali or acid which can be discriminated in practice by titrating with the burette. If we consider the titration of 1 ml. of $N/10$ acid delivered for example from a 1 ml. simple pyrex pipette (drawn out tube—length 17·5 cm.) the coefficient of variation in millimols of KOH is 25×10^{-5}. Where $N/100$ fluids are used the standard deviation in millimols is ten times less, and further reduction in the strength of the fluids will progressively reduce the standard deviation expressed in absolute units. We reach a point however when the standard deviation so

expressed becomes constant. This limit of standard deviation sets the limit of our discrimination and is proportional to the total buffering power of the fluid at the end multiplied by the indiscernible range here of the p_H figure. The limit is reached for the present horizontal burette when the quantity of acid titrated is in the region of 10^{-4} millimol.

Table I shows the first seven of a series of 20 titrations of 1 ml. of acid-indicator mixture of strength 0·0002 *N* with 0·001 *N* carbon dioxide-free alkali. The acid was of a kind previously described [Conway and Byrne, 1933] containing 20 % alcohol, 0·0008 % methyl red and 0·00016 % methylene blue. The standard deviation in millimols was found to be $1{\cdot}7 \times 10^{-6}$. The total quantity of fluid at the end-point was 1·2 ml. contained in about 1 ml.

Table I.

No. of titration	Quantity of acid millimols	Burette reading (0·001 *N* KOH) cm.	Burette reading as percentage of mean reading
1	$10^{-3{\cdot}7}$	25·6	101·2
2	,,	25·2	99·6
3	,,	25·5	100·8
4	,,	25·1	99·2
5	,,	25·3	100·0
6	,,	25·3	100·0
7	,,	25·5	100·8

The above figures give the results of the first seven titrations of a series of 20. In each case 1 ml. of 0·0002 *N* sulphuric acid containing 20 % alcohol, 0·0008 % methyl red, and 0·00016 % methylene blue, was titrated. The pyrex pipette used for delivering the acid delivered with a coefficient of variation of 0·16 %. The coefficient of variation of the individual titration was 0·85 %. The widest variations in the series were 1·6 and −2·0 % of the mean representing 3·2 and $-4{\cdot}0 \times 10^{-6}$ millimol respectively. The mean error of the burette reading on a single determination was 1·85 mm. This represents $1{\cdot}48 \times 10^{-6}$ of a millimol and corresponds almost exactly to the error described for the Rehberg burette [Rehberg, 1925].

For titrations in atmospheric air it is not possible to diminish this figure appreciably for the above volume by titrating closer to a p_H of 7·0, for any diminution of the buffering power of the water secured thereby is more than counterbalanced by the rapidly increasing buffering power of the carbon dioxide of the air as well as by the instability of the end-point. The carbon dioxide of the air will be in equilibrium with distilled water of approximately p_H 5·6, so that the methyl red indicator has the great advantage that its point of maximum sensitivity lies in the same region.

In a carbon dioxide-free atmosphere and titrating to p_H 7·0 with the above glassware and similar fluids and volumes it is possible to decrease the standard deviation from $1{\cdot}7 \times 10^{-6}$ to $0{\cdot}5 \times 10^{-6}$ millimol KOH, provided we substitute for the indicator mixture one equally sensitive at a p_H of 7·0 and provided also our alkali is perfectly carbon dioxide-free.

The horizontal micro-burette has been found of value in the following procedures.

(1) It may be substituted for the Bang burette over the whole range of micro-titration, being more accurate for the lower ranges (10^{-3} m.eq. or less with acidimetric titration) and of the same accuracy for the higher. It possesses the added advantage of permitting the use of standard solutions ten times more concentrated. The 100 ml. storage bottle will permit the easy filling and delivery of 50 cm. length of the graduated section 250 times without any contact with the carbon dioxide of the atmosphere. The graduated section alone need be cleaned for a smoothly running meniscus.

(2) In the micro-titration of ammonia and urea as described in a previous communication [Conway, 1933] it can be substituted advantageously for the Bang 2 ml. burette. For example in the estimation of urea in 0·2 ml. of blood 1 ml. of 0·1 N sulphuric acid may be placed in the central chamber of the absorbing unit and titrated with 0·025 N carbon dioxide-free alkali. 50 cm. length of alkali will titrate the acid (otherwise a factor is introduced) and the calculation is simply the multiplication by 3 of the cm. of alkali on the graduated tube corresponding to the urea-ammonia. This gives the result in mg. urea per 100 ml. blood.

(3) The burette has been used for the estimation of ammonia in normal blood in conjunction with the absorbing unit described in a previous communication [Conway, 1933] and according to a procedure to be subsequently described.

(4) It has been used for the micro-titration of benzidine sulphate precipitates in centrifuge-tubes in the manner described by Cope [1931] with the Rehberg burette.

(5) It is useful in various micro-thiosulphate titrations permitting the use of comparatively concentrated standard solutions.

(6) It can be used for the very accurate delivery of small volumes of fluid of which a comparatively large quantity is available. It will deliver 0·1 ml. with from one-half to one-third the error of an Ostwald pipette, and more accurately than the ordinary analytical balance can be relied upon to weigh.

(7) It may be used as a very accurate wash-out pipette for very small volumes of blood or serum. In using it for this object water is sucked up in tube F (Fig. 1) to the highest level, the meniscus in the graduated tube being previously adjusted to some point near the end of the graduations. On opening the delivery tap there is now a suction pressure created which may be used to suck in a volume of blood which will be registered by the movement of the meniscus in the graduated tube. The exact volume taken in is thus measured and may then be delivered with an exact volume of wash-out water. It will be seen that this method is very convenient for small volume measurements which need not be exactly represented by tenths of a ml.

Summary.

A simple micro-burette is described which can be easily assembled from pyrex thermometer tubing and graduated tubing. It delivers volumes of the order of 0·1 ml. and may be used for all micro-titrations. It is particularly suitable for titrating quantities of acid of the order 10^{-6} equivalent or less. Its error for delivering 0·1 ml. expressed as a coefficient of variation is 0·12 %. Its error for titrating 1 ml. of 0·0002 N sulphuric acid is $1{\cdot}7 \times 10^{-6}$ expressed as a standard deviation in millimols of alkali.

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XL. VITAMIN A AND CAROTENE.

XI. THE DISTRIBUTION OF VITAMIN A IN THE ORGANS OF THE NORMAL AND HYPERVITAMINOTIC RAT.

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(*Received January 6th, 1934.*)

In a previous paper Moore [1931] described the distribution of vitamin A and carotene, as determined by colorimetric methods, in the organs of rats which had received large amounts of carotene as red palm oil or carrot-fat. It was found on the one hand that unchanged carotene persisted throughout the alimentary tract, while on the other a high concentration of vitamin A accumulated in the liver. Outside the liver only small amounts of vitamin were detected. Thus the concentration in the storage fat was only about 1/100,000 of that found in the liver-fat, while in most other tissues, which of course contained only small percentages of fat, the amounts of vitamin present were too small to be detected by colorimetric means. In agreement with previous biological experiments by Sherman and Boynton [1925] however and with the subsequent work of Simmonet and Busson [1932] the kidney and lungs gave faintly positive reactions in some cases. It was concluded that the liver must not only be able to store vitamin A to a much greater extent than any other organ, but must also in a complementary sense play a large part in keeping the concentration of vitamin A throughout the rest of the body within prescribed limits. This question was discussed further in the case of the cow [Moore, 1932, 1].

The experiments described below were carried out as an extension of the previous work in order to keep pace with recent developments in our knowledge of the physiological behaviour and significance of vitamin A as studied from other angles. In the first place work on the vitamin A of human livers obtained at autopsy has shown that chronic nephritis is one of the most noteworthy of those diseases which are characterised by the occurrence of a high proportion of cases having subnormal vitamin A reserves [Wolff, 1932; Moore, 1932, 2], while the same tendency, although to a much less well marked extent, is noticed in bronchitis and pneumonia [Moore, 1932, 2; Fox, 1933]. It is therefore of interest to decide whether measurable amounts of vitamin A are normally present in the kidneys and lungs, and how far these organs are to be differentiated from others in this respect. Secondly it seemed desirable to extend the previous work to include rats which had received large amounts of vitamin A in place of carotene. Finally the ready availability of comparatively pure preparations of vitamin A has led to the confirmation by many workers of Takahashi's original finding that excess of vitamin A has a toxic action [Takahashi *et al.*, 1925; Harris and Moore, 1928; 1929; Drigalski, 1933; Moll *et al.*, 1933; Bomskov and Seemann, 1933;

Collazo and Rodriguez, 1933]. Knowledge of the distribution of vitamin A in the organs of rats suffering from hypervitaminosis must obviously be of interest as a step towards the elucidation of the cause of the toxic action.

Experimental.

Vitamin A was assayed colorimetrically, using the alkali digestion technique [Davies, 1933], in the organs of albino rats which had been given varying amounts of carotene or vitamin A for varying periods. In the case of small organs, or organs containing small amounts of vitamin, the fat obtained after digesting the tissues was made up in 1 or 2 ml. of chloroform instead of in the 5 ml. usually employed in routine work.

The presence of vitamin A in the kidneys of rats having moderate liver reserves.

Five rats which had been used in vitamin A tests, and which presumably possessed negligible vitamin A reserves at the end of the test period, were given for several weeks a diet containing carotene as 15 % of red palm oil, with the addition of carrots in some cases. Vitamin A was assayed in the liver, kidneys, lungs and (in 3 cases) the intraperitoneal fat. The results are given in Table I.

Table I. *Rats having moderate liver reserves.*

	Rat 1 ♂		Rat 2 ♂		Rat 3 ♂		Rat 4 ♂		Rat 5 ♂	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	900	90	1200	150	1500	150	1000	100	500	50
Kidneys	10	5	6	3	4	3	2	1	8	3
Lungs	6	3	>3	>1·5	0	0	0	0	0	0
Intraperitoneal fat	—	—	—	—	—	0	—	0	—	2

It will be seen that the dietary treatment resulted in the accumulation of moderate vitamin A reserves in the liver[1]. Under these circumstances small amounts of the vitamin were detected in the kidneys in all cases. Only in two out of five was a positive reaction given by the lungs, and in one case out of three by the intraperitoneal fat.

The distribution of vitamin A in the organs of rats given large, but non-toxic, doses of vitamin A concentrate.

Three adult rats were given about 3·5 mg. of B.D.H. vitamin A distillate (3000 B.U. per mg.) each daily for 38 days. They were anaesthetised and killed by bleeding. In order to facilitate the detection of vitamin A in tissues expected to contain only small amounts, the various organs from all three rats were combined after dissection. Results are shown in Table II.

In these rats the liver reserve was extremely high, being 160 times greater than the normal human reserve. The concentration in the kidneys was about 10 times greater than in the previous experiment, but even greater concentrations were found in the lungs and suprarenals, in both of which values were observed

[1] For purposes of comparison it may be mentioned that the "normal" human liver reserve in this country, taken as the median value over a wide range of cases dying by accident, is about 250 B.U. per g. A similar value was obtained in the case of a single wild rat shot in the vicinity of this laboratory.

Table II. *Rats given large, but non-toxic, amounts of vitamin A concentrate.*

Combined tissues from 3 adult ♀ rats, Nos. 6, 7, 8.

	Total B.U. per rat	B.U. per g.
Liver	300,000	40,000
Kidneys	70	50
Lungs	500	450
Suprarenals	100	2,500
Heart	1	1
Spleen	1	2
Pancreas	15	25
Thymus	4	12
Brain	0·5	0·3
Muscles	—	0·5
Blood	—	2
Intraperitoneal fat	—	80

which would be considered quite high for the liver in normally fed rats. In the brain, heart, spleen, muscles and blood only small traces of vitamin were detected, but slightly larger amounts seemed to be present in the pancreas, thymus and intraperitoneal fat. The value in the case of the pancreas may have been made unduly high through the inadvertent inclusion of fat deposits.

To obtain further evidence on the points of interest raised in this experiment further assays were carried out on the liver, kidneys, lungs, pancreas and suprarenals of four adult rats which had received large amounts of vitamin A concentrate. These animals differed from those just described in so far that the feeding of concentrate was discontinued some weeks before killing, an ordinary stock diet of bread and milk, cereals and vegetables being given in the meantime. The organs from each rat were kept separate. Results are given in Table III.

Table III. *Rats given large, but non-toxic, doses of vitamin A concentrate. Concentrate feeding discontinued some time before killing.*

	Rat 9 ♀		Rat 10 ♂		Rat 11 ♀		Rat 12 ♂	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	75,000	8,000	150,000	15,000	125,000	15,000	125,000	10,000
Kidneys	10	5	10	5	15	10	5	2
Lungs	250	150	60	35	75	50	125	60
Pancreas	0	0	5	5	0	0	0	0
Suprarenals	0	0	2·5	25	5	70	1·5	15

In most cases the concentration of vitamin A in the liver was about half that observed in the preceding experiment. The concentration in the kidneys was remarkably low, being little in excess of that found in the case of the rats having moderate reserves. In the lungs the concentration was again greater than in the kidneys but was much lower than the value found in the previous experiment. The amounts of vitamin present in the suprarenals were much smaller than in the preceding experiment, while in the pancreas a negative result was obtained in three rats, a trace being found in the case of one animal.

The distribution of vitamin A in the organs of rats given toxic doses of vitamin A.

Two young albino rats were given the following diet. "Light white casein" 20 %; rice starch 35 %; coconut oil 40 %; salt mixture 5 %; dried yeast 10 % (additional); radiostol (vitamin D) 1 drop per week. Vitamin A concentrate was given dissolved in the coconut oil at such a concentration that 40 mg. were

included in every 10 g. of diet. To avoid undue loss of the vitamin before ingestion by the rat the solution of the vitamin in the oil was stored separately and admixed with the remaining components of the diet daily immediately before feeding. The history of the rats was as follows.

Rat 13 ♂. Initial weight 95 g. Declined in weight from commencement of the experiment with the development of a hunched attitude, loss of hair round the mouth[1] and haemorrhagic rhinitis. Died after 12 days, final weight 60 g. Average daily food consumption 3·1 g.; consumption of concentrate 12·5 mg. per day. Total concentrate ingested, 150 mg. *Post mortem* examination revealed extreme emaciation and severe lung disease. Parts of the lungs were red, wet and soft, other parts were filled with putty-like hard nodules which were present in all sizes up to about 7 mm. in diameter.

Rat 14 ♀. Initial weight 81 g. Declined in weight from commencement with superficial appearances similar to those observed in the previous rat. Died after 33 days, the final weight being 55 g. Average daily consumption of food 3·6 g., of concentrate 14·3 mg. Total concentrate ingested 471 mg. At autopsy the lungs appeared slightly reddened but did not exhibit the gross lesions observed in the preceding rat. The immediate cause of death appeared to be extreme emaciation. The most striking feature, however, was the extreme softness of the bones. X-ray examination revealed that several of the larger leg bones had sustained spontaneous fractures, the broken ends of the bones being healed together in irregular shapes with the formation of calluses.

Table IV. *Rats given toxic amounts of vitamin A concentrate.*

	Rat 13 ♂		Rat 14 ♀	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	50,000	15,000	200,000	60,000
Kidneys	40	60	500	600
Lungs	2,500	1,250	1,000	1,000
Suprarenals	0	0	2·5	50
Heart	60	100	15	25
Spleen	0	0	2·5	15
Pancreas	0	0	1	10
Brain	25	15	20	15
Eyes	0	0	0	0
Testicles	0	0	—	—
Muscles	—	0	—	25
Bone	—	0	—	2
Total B.U. ingested	450,000	—	1,400,000	—
Found in faeces	1,500	—	6,400	—
Found in urine	0	—	0	—

The results of vitamin A assays carried out on the organs of these rats are given in Table IV. In the case of the rat dying after 12 days the liver reserve was considerably below the values obtained in some other cases. The lungs contained a very high concentration of vitamin, which in view of the diseased and unduly weighty condition is best judged in this case from the total vitamin present rather than the value expressed per g. of tissue. Considerable amounts of vitamin were present in the kidney and heart. A small amount was found in the brain. Other organs gave negative results.

[1] The loss of hair round the mouth would appear to be due to the irritant action of traces of diet remaining on the skin after feeding. It is noteworthy, however, that the concentrate exerts no immediate irritant action. Our solution of the vitamin A concentrate in coconut oil could be eaten by ourselves in small amounts without irritation or discomfort.

In the case of the rat dying after 33 days the concentration of vitamin A in the liver was four times greater than in the preceding rat. The total amount of vitamin present in the lungs was less, but in this case a high value was found for the kidneys. Significant amounts of vitamin were also found in all organs except the eyes[1].

In order to determine the efficiency of the absorption of vitamin A in these rats the faeces and urine were collected daily and stored in 5 % KOH until the end of the experiment, when vitamin A assays were carried out on aliquot portions by the usual technique. In each case the urine gave a negative result. Positive results were found for the faeces, but the amounts present were insignificantly small when compared with the difference between the amount of vitamin ingested and that found in the body. In each case only about $\frac{1}{7}$ of the ingested vitamin could be accounted for, the remainder presumably being lost by oxidation at some stage as yet unknown.

The distribution of vitamin A in the organs of rats given large doses of carotene.

Two albino rats from the same litter as those used in the preceding experiment were given the same diet except for the substitution of carotene (B.D.H.) in place of vitamin A. Owing to the limited solubility of the pigment it was found impossible to incorporate more than 10 mg. per 10 g. of diet (*i.e.* $\frac{1}{4}$ of the concentration of vitamin A in the preceding experiment), but since the food consumption was much greater the actual daily consumption of carotene was about half that of vitamin A. The animals grew rapidly and when killed after 34 days were found at autopsy to be perfectly normal. The storage fat was plentiful and quite colourless. Particulars were as follows.

Rat 15 ♂. Initial weight 95 g. Final weight 156 g. Average daily food consumption 8·3 g.; carotene consumption 8·3 mg. daily; total carotene ingested 281 mg.

Rat 16 ♀. Initial weight 83 g. Final weight 129 g. Average daily food consumption 7·9 g.; carotene consumption 7·9 mg. daily; total carotene consumption 268 mg. The distribution of vitamin A in the organs of these rats is shown in Table V.

Table V. *Rats given large amounts of carotene.*

	Rat 15 ♂		Rat 16 ♀	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	12,500	1,250	12,500	1,700
Kidneys	5	4	5	3
Lungs	30	30	20	12
Suprarenals	0	0	0	0
Heart	0	0	0	0
Spleen	1·5	3	0	0
Pancreas	2·5	10	0	0
Brain	0	0	0	0
Eyes	0	0	0	0
Testicles	0	0	—	—
Muscles	—	0	—	0
Bone	—	0	—	0
Intraperitoneal fat	—	5	—	2
Y.U. carotene ingested	560,000	—	530,000	—
Found in faeces	55,000	—	25,000	—

[1] In view of the fact that the retina has been reported to contain considerable amounts of vitamin A [Smith *et al.*, 1931; Wald, 1933] this negative result should not be taken to imply that the vitamin was entirely absent, but it is evident that the concentration of the vitamin, if present, was not sufficiently high to compensate for the small amounts of tissue available for assay.

The vitamin A reserves found in the liver would represent high values for ordinary conditions of nutrition but are some 30 times lower than the maximum value obtained by giving vitamin A. In the kidney the concentration of vitamin was no greater than in the rats having only moderate liver reserves, but in the lungs the amounts present were of a slightly higher order. In each case a faintly positive reaction was given by the intraperitoneal fat, while in rat 15 traces of chromogen were also detected in the spleen and pancreas. In the case of the spleen the yellow/blue ratio indicated that the reaction was due to carotene.

In order to determine the efficiency of absorption of the carotene the faeces and urine were examined as in the preceding experiments, the amounts found representing about 10 % and 5 % of the ingested pigment in rats 15 and 16 respectively. Since the amount of vitamin A present in the body must in each case have represented only some 3–6 mg. of carotene, or about 1–2 % of the ingested carotene, it is obvious that the utilisation of carotene at such high levels must be very inefficient, and that the greater part of the pigment has not been accounted for.

Discussion.

The distribution of vitamin A in the organs of the rat.

The above experiments indicate that small amounts of vitamin A (*ca.* 5 B.U. per g.) are consistently present in the kidneys even when the liver reserves are of a moderate order (*ca.* 100 B.U. per g.). Traces of the vitamin are sometimes present in the lungs under these conditions.

When the liver reserve is raised by the inclusion in the diet of large amounts of carotene or vitamin A the order of the kidneys and the lungs is reversed, since the lungs now invariably show a greater concentration of vitamin A. As far as can be judged both from the present experiments and from previous work [Moore, 1931] carotene feeding does not result in the accumulation of vitamin A in high concentration in any organ except the liver, the highest value observed being 30 B.U. per g. for the lungs of rats Nos. 15 and 16 in the present work. The feeding of vitamin A concentrate on the other hand may raise the concentration in the kidneys and lungs to values that would be high even for the liver in the case of animals receiving ordinary diets. At the same time appreciable amounts of vitamin may appear throughout the whole body. In the case of the suprarenal glands very inconsistent results have been obtained. For rats 6, 7 and 8 a very high average value (2500 B.U. per g.) was observed, but in other experiments the results were much lower and in some cases negative. It may be noted that in view of the small size of these organs the actual amount of vitamin involved was relatively small.

Hypervitaminosis A.

The production of toxic symptoms in the present work may be considered to provide further evidence as to the reality of hypervitaminosis A in so far as the preparation used was a distillate, as opposed to the non-distilled concentrates used, as far as we are aware, by other workers in this field. Drigalski [1933] has already shown that the toxic power of the concentrates is destroyed with the vitamin on ultra-violet irradiation, it is now evident that the vitamin potency and toxic power are stable to distillation and are not separated by this means. Although only two rats were used in the present work we were fortunate in observing almost all the symptoms described by previous workers. The lung lesions observed in rat 13 exactly resembled those described in several cases by Takahashi *et al.* [1925]. It is perhaps noteworthy that these lesions should occur

in the organ in which the greatest concentration of vitamin A outside the liver was located. In rat 14 the bone lesions were exactly similar to those described by Bomskov and Seemann [1933] and also Collazo and Rodriguez [1933], our own observations representing the third independent discovery of the phenomenon.

Speculation as to the mechanism of the production of hypervitaminosis A would be premature at the present stage, but two clearly established points may be worth mention. In the first place it is obvious that the liver possesses a very high toleration for the vitamin, since concentrations of the order observed in the hypervitaminotic rats (Nos. 13 and 14) were also observed in healthy animals (Nos. 6, 7 and 8). At the maximum level observed the vitamin must have constituted about 5 % of the dry weight of the liver and have represented a supply sufficient to last the rat for about 200 years if economically used. Secondly it is plain that in the case of rat 13 death through hypervitaminosis intervened at a point when the liver still contained less than half the maximum observed concentration of vitamin. This finding shows that death may occur while there is still room for a considerably greater accumulation of vitamin A in the liver and suggests that the production of hypervitaminosis does not depend on the absolute amount of vitamin A present in the organism but on the ingestion of the vitamin at a greater rate than that at which it can be stored by the liver or eliminated by the organism.

The absence of toxic symptoms after feeding excess of carotene.

The absence of any symptoms of hypervitaminosis in the rats (Nos. 15 and 16) given about 8 mg. of carotene daily suggests that it is virtually impossible to induce hypervitaminosis A by carotene feeding. This conclusion has already been reached by Kuhn and Brockmann [1933], but since these workers administered carotene at the rate of only about 1 mg. per day, which is little greater than the dosage of 0·75 mg. used by Moore [1930] in earlier experiments, it is obvious that further evidence on this point was desirable.

It is still possible, of course, that large masses of solid carotene, as opposed to solutions of carotene in oil, might possess toxic action. Even if this proved to be the case, however, the phenomenon observed would almost certainly be quite different from the hypervitaminosis induced by giving vitamin A concentrate, since a comparison of the vitamin A contents of the livers of rats 13 and 14, which were given large amounts of vitamin A concentrate, with those of rats 15 and 16, which were given large amounts of carotene, indicates that the rate at which the liver reserve can be raised by the conversion of carotene (50 B.U. per g. per day) is much slower than the rate of increase obtained by giving vitamin A (1200–1700 B.U. per g. per day). The superior rate and efficiency of absorption of vitamin A suggest that in intensive vitamin A therapy preformed vitamin A should be more efficacious than carotene. At levels approaching the minimum dose, as previously shown [Moore, 1933], vitamin A and carotene appear to be equally efficiently utilised.

Summary.

1. Vitamin A was assayed by the antimony trichloride method in the organs of rats which had received varying amounts of vitamin A or carotene.

2. Even when the liver reserves were quite low (100 B.U. per g.) small amounts of vitamin A (5 B.U. per g.) were found in the kidneys and sometimes in the lungs.

3. When large, but non-toxic, doses (3·5 mg. daily) of vitamin A distillate were given to adult rats (*a*) the concentration of vitamin in the liver rose to

extremely high levels (40,000 B.U. per g.), (*b*) the concentration in the lungs was of the order found in the liver under normal conditions of nutrition, (*c*) the concentration in the kidney remained fairly low (50 B.U. per g.), (*d*) the concentration in other organs was extremely low, except that surprisingly high values were observed in the suprarenals (up to 2500 B.U. per g.).

4. Even more massive doses of the distillate (12–14 mg. per day) given to young rats proved toxic, causing emaciation, haemorrhagic rhinitis, loss of hair round the mouth and (*a*) in one rat, dying after 12 days, gross lung lesions, (*b*) in another rat, dying after 33 days, a peculiar softening and hollowing of the bones which had resulted in spontaneous fractures of the legs. The distribution of vitamin A resembled that found in rats given non-toxic doses except that the concentration in the lungs was even higher (1250 B.U. per g.), while in the rat dying after 33 days a high kidney value (625 B.U. per g.) was observed.

5. Attempts to induce hypervitaminosis A by feeding carotene failed. On account of its limited solubility in fats only 8 mg. of the pigment could be given daily. Rats receiving this amount grew rapidly and remained in perfect health. On killing after 34 days the liver reserve was found to be only moderately high (1250 B.U. per g.) pointing to only about 1 % absorption, as opposed to 15 % in the case of vitamin A. Only very small amounts of vitamin were present in the lungs and kidneys, negative results being obtained in all other organs.

Our thanks are due to Dr L. J. Harris for his valuable criticism.

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XLI. THE INACTIVATION OF ANIMAL AMYLASE BY PLANT PARALYSERS AND THE PRESENCE OF INACTIVATING SUBSTANCES IN SOLUTIONS OF ANIMAL AMYLASE.

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(*Received January 8th, 1934.*)

In the preceding papers [1933] it was shown that in germinated and ungerminated cereals there are natural paralysers of amylase, which we call "sistoamylase substances." Buckwheat malt contains many of these paralysers, mostly in the leaves and roots. The sistoamylase adsorbs the plant amylase either in a state of solution or at the moment of being dissolved and so inactivates it.

Moreover we have found other substances, which counteract the sistoamylase and have named these substances "eleutoamylases." Any eleutoamylase added to the system sistoamylase-amylase prevents inactivation by the paralyser, or if the amylase has been already inactivated by the sistoamylase, the whole or a greater part of the inactivated amylase is put into action by the eleutoamylase. The most active eleutoamylases recognised by us are products of decomposition of protein found in peptone.

In the present paper the same problem is discussed in relation to animal amylase. Before anything else is suggested the following questions should be answered:

(1) Is plant sistoamylase able also to inactivate animal amylase?

(2) Does the system composed of plant sistoamylase and animal amylase react like the similar system containing plant amylase, the amylase of which can be reactivated by adding a suitable eleutoamylase?

(3) Do there co-exist in the animal organism besides amylases sisto-substances characterised by properties similar to those of the plant sistoamylases?

Experimental.

The experiments were conducted on human saliva, collected on the day of starting experiments, and on Merck's pancreatic amylase. The saliva was diluted in the proportion 5:100, well mixed during 5 mins. and filtered. The solutions of pancreatic amylase were prepared by dissolving 0·25 g. of pancreatic amylase in 100 ml. of water at 20°, shaken during 5 mins. and then filtered. The leaves and roots of buckwheat-malt, germinated within 15 days at 15°, were taken as material containing the plant paralyser, the sistoamylase substance. This material was used in two different manners: well crushed undried, and dried in the apparatus of Faust-Heim at 35° and then reduced to powder. "Peptone siccum sine sale" (Merck) served as eleutoamylase. The p_H of Merck's soluble starch solution was adjusted by additions of phosphate buffers to 6·7, determined

potentiometrically. The amylolytic activities of the solutions of amylase were measured by their liquefying, dextrinising and saccharifying powers.

The liquefying power of saliva was determined with native starch according to the method of Lintner-Sollied [1902], but at 40° instead of 65°. The liquefying power of pancreatic amylase was established by the method of Pollak [1903] at 40°.

The dextrinising power. For pancreatic amylase a 2·5 % and for salivary amylase a 1 % solution of Merck's soluble starch was prepared, by addition of starch paste to boiling water, boiling the solution for a further 3 mins., and, after cooling it, adjusting the p_H. 50 ml. of this solution were warmed for 30 min. at 40°, afterwards 5 ml. of the enzyme solution to be tested were introduced. At intervals of 4 mins., 1 ml. of this system was taken and added to 1 ml. of iodine solution (0·8 g. iodine in 1 litre H_2O) and the colour obtained was observed. The dextrinising activity was measured by the time required for obtaining the following colours: violet, brownish red, light yellow.

The saccharifying power was investigated with the same solutions of soluble starch also at 40°; 5 ml. of the enzyme solution were added to 50 ml. of the solution of soluble starch and treated as before. After 15 mins. 10 ml. of $N/10$ NaOH were added, the mixture was cooled, and reducing sugars were determined in 30 ml. by the method of Willstätter and Schudel [1918]. The saccharifying power is expressed by the number of ml. of $N/20$ iodine reduced by 30 ml. of the liquid tested. The amount of iodine reduced by the control test was deducted.

I. Salivary amylase.

(a) *Inactivation of salivary amylase by plant paralyser.*

In order to ascertain whether the plant paralyser also inactivates the amylase of saliva and in what degree, plant paralyser was taken from two sources: dried leaves and rootlets of buckwheat-malt (1) obtained a year ago and (2) obtained shortly before our experiments. The mixtures used were prepared by addition of the quantities of sistoamylase substance shown in Table I to 40 ml. of 5 % saliva. In each case (except No. 1) the solution was shaken mechanically for 8 mins. and then filtered, and the amylolytic activity of the filtrate was investigated (Table I).

Table I.

No. of solution	Additions g.	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet	Brownish red	Light yellow (was obtained with iodine in mins.)	Liquefying power Lintner-Sollied method 1 g. starch is liquefied by 5 % saliva, ml.
1	None	1·8	20	40	220	1·8
2	0·1 sistoamylase 1	0·1	110	180	Not obtained in 240 mins.	2·4
3	0·3 ,,	0·1	Without change after 240 mins.			Not liquefied by 4 ml.
4	0·5 ,,	0·1	,,			,,
5	0·7 ,,	0·1	,,			,,
6	0·1 sistoamylase 2	0·1	190	Not obtained after 240 mins.		,,
7	0·3 ,,	0·1	Without change after 240 mins.			,,
8	0·5 ,,	0·1	,,			,,
9	0·7 ,,	0·1	,,			,,

These experiments demonstrate that a plant paralyser also inactivates salivary amylase: 0·3 g. of sistoamylase substance is sufficient to inactivate completely 40 ml. of 5 % saliva in 8 mins. The dried sistoamylase substance obtained shortly before the experiment possesses the inactivating power in a higher degree than that obtained a year ago.

(b) The inactivation of salivary amylase by sistoamylase substance in presence of peptone.

(1) *Peptone an antisistoamylase substance to salivary amylase.* The next point which we investigated was the inactivation of salivary amylase by a dried paralyser in presence of peptone. Peptone was added simultaneously with the sistoamylase to the solution of salivary amylase. The solutions used were made up by addition of the substances shown in Table II to 40 ml. of 5 % saliva. In each case the solution was mechanically shaken for 8 mins. and then filtered, and the amylolytic action of the filtrate was investigated (Table II).

Table II.

No. of solution	Additions: Peptone g.	Additions: Sistoamylase substance g.	Saccharifying power ml. N/20 I	Dextrinising power: Violet	Dextrinising power: Brownish red	Dextrinising power: Light yellow	Liquefying power Lintner-Sollied method 1 g. starch is liquefied by 5 % saliva, ml.
				was obtained with iodine in mins.			
1	None	None	1·6	60	90	240	1·8
2	0·5	,,	4·5	10	19	84	1·2
3	1·0	,,	3·9	10	16	66	0·9
4	2·0	,,	3·5	9	15	54	0·9
5	None	0·3 Prep. 1	0·1	Without change after 4 hours			Not liquefied by 4 ml.
6	,,	0·3 ,, 2	0·1	,,	,,	,,	,,
7	0·5	0·3 ,, 1	2·0	15	30	130	1·2
8	1·0	0·3 ,, 1	3·8	14	21	84	1·0
9	2·0	0·3 ,, 1	3·1	10	17	60	1·0
10	0·5	0·3 ,, 2	2·6	18	32	132	2·1
11	1·0	0·3 ,, 2	3·8	12	21	84	1·0
12	2·0	0·3 ,, 2	3·0	11	18	58	1·0

The experiments demonstrate that treatment with peptone greatly increases the amylolytic activity of salivary amylase. This fact can be explained on the ground that in saliva only a small quantity of the amylase is active. The greater part is inactivated, and its activity can be restored by addition of peptone. Evidently in the diluted saliva there must be an inactivating substance with the properties of our sistoamylase. It is shown later that the plant paralyser does not inactivate salivary amylase in the presence of peptone. These results are in agreement with those obtained for plant amylase. It is interesting that peptone has a different effect on the liquefying, dextrinising and saccharifying powers of salivary amylase.

(2) *Peptone an eleutoamylase substance to salivary amylase.* In order to ascertain whether eleutoamylase restores the activity of salivary amylase which has been inactivated by the plant paralyser (dried or undried) the following solutions were used.

No. of solution															
1	40 ml. 5 % saliva	...	...	...	...	...	...	...	...	...	...	...	...	...	filtered
2	,,	+0·3 g. dried sistoamylase.				(1)	Shaken	10	mins. and then	+2 g. peptone, eluted		15	mins. then	filtered	
3	,,	,,		,,		,,	,,	10	,,	,,		30	,,	,,	
4	,,	,,		,,		,,	,,	10	,,	,,		60	,,	,,	
5	,,	,,		,,	(2)	,,	,,	10	,,	,,		15	,,	,,	
6	,,	,,		,,		,,	,,	10	,,	,,		30	,,	,,	
7	,,	,,		,,		,,	,,	10	,,	,,		60	,,	,,	
8	,,	+1 g. undried		,,		,,	,,	8	...	...	...	...	...	then	,,
9	,,	,,		,,		,,	,,	8	,,	+2 g. peptone, eluted		10	mins. then		,,
10	,,	,,		,,		,,	,,	15	,,	,,		10	,,	,,	,,
11	,,	,,		,,		,,	,,	30	,,	,,		10	,,	,,	,,
12	,,	,,		,,		,,	,,	60	,,	,,		10	,,	,,	,,
13	,,	,,		,,		,,	,,	8	,,	,,		30	,,	,,	,,
14	,,	,,		,,		,,	,,	15	,,	,,		30	,,	,,	,,
15	,,	...	...	...	...	...	...	...	...	+2 g. peptone, shaken		8	,,	,,	,,

The amylolytic action of the filtrates was then investigated.

Table III.

No. of solution	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet was obtained with iodine in mins.	Dextrinising power: Brownish red was obtained with iodine in mins.	Dextrinising power: Light yellow was obtained with iodine in mins.	Liquefying power Lintner-Sollied method 1 g. of starch is liquefied by 5 % saliva, ml.
1	1·6	60	90	240	1·8
2	0·1	Without change after 4 hours			Not liquefied by 4 ml.
3	0·1		,,		,,
4	0·1		,,		,,
5	0·1		,,		,,
6	0·1		,,		,,
7	0·1		,,		,,
8	0·0		,,		,,
9	1·7	24	42	120	1·2
10	1·0	26	54	Not obtained	1·3
11	0·6	48	90	,,	1·4
12	0·6	90	174	,,	1·7
13	1·7	24	40	124	0·9
14	1·1	34	48	218	1·1
15	5·8	9	12	42	0·3

The dried plant paralyser inactivates the amylolytic action of salivary amylase so strongly that even prolonged contact of peptone with adsorbed amylase does not lead to an elution of the ferment, and such a system does not exhibit amylolytic activity. After treatment with undried plant paralyser on the other hand peptone reactivates the salivary amylase. These results are in agreement with those obtained on plant amylase.

It remains to be established whether the addition of peptone to the system undried sistoamylase + salivary amylase, the amylolytic action of which is nearly nil, actually leads to elution of amylase or whether a small part of the amylase undergoes no adsorption and gives the corresponding intensive amylolytic action with peptone. To this end, the following solutions were prepared. To 40 ml. of 5 % saliva was added 1 g. undried sistoamylase, the mixture shaken for a definite time and filtered during 6 mins. The nature of the sistoamylase added and the times of shaking are shown below.

No. of solution	Duration of malting in prep. of sistoamylase days	Duration of shaking mins.
1	11	8
2	11	12
3	11	27
4	11	57
5	25	8
6	25	27
7	25	57

Two series of experiments were made by establishing the dextrinising and saccharifying powers of each of the above solutions after filtration. The first series (*a*) without any addition, the next (*b*) with the addition of 0·2 g. peptone to 50 ml. of 1 % soluble starch solution. If peptone does not elute but only increases the amylolytic activity of the inactivated solutions, the numbers of series "*b*" must agree with the results in solutions 9–14 in Table III.

In Table IV we see that the young undried sistoamylase substance produced in 11 days' malting adsorbs the salivary amylase quantitatively. But the

Table IV.

No. of solution	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet (was obtained with iodine in mins.)	Brownish red (was obtained with iodine in mins.)	Light yellow (was obtained with iodine in mins.)	Liquefying power Lintner-Sollied method 1 g. of starch is liquefied by 5 % saliva, ml.
1 *a*	0·0	Without change after 4 hours			Not liquefied by 4 ml.
1 *b*	0·0	,,			,,
2 *a*	0·0	,,			,,
2 *b*	0·0	,,			,,
3 *a*	0·0	,,			,,
3 *b*	0·0	,,			,,
4 *a*	0·0	,,			,,
4 *b*	0·0	,,			,,
5 *a*	0·0	,,			,,
5 *b*	0·1	,,			,,
6 *a*	0·0	,,			,,
6 *b*	0·1	120	180	Not obtained	,,
7 *a*	0·1	Without change after 4 hours			,,
7 *b*	0·1	110	160	Not obtained	,,

undried sistoamylase substance produced in 25 days also adsorbs the amylase at first very strongly; however, shaking the system for a long time has the effect that a small quantity of the sistoamylase with adsorbed amylase passes into solution. This dissolved system does not itself show any amylolytic action, but when peptone is added, as in the experiment with the solutions of starch of series "*b*," this system begins to act, the action of the sistoamylase being eliminated.

II. Pancreatic amylase.

(*a*) *Inactivation of pancreatic amylase by plant paralyser.*

To ascertain whether the plant sistoamylase is able to inactivate pancreatic amylase, the following experiments were made. Solutions of 0·25 g. pancreatic amylase in 100 ml. of water were shaken mechanically for 5 mins. and then various quantities of sistoamylase were added, the solutions shaken for 8 mins. and filtered. The amylolytic power of the filtrates was then examined (Table V).

Table V summarises the results.

Table V.

No. of solution	Additions g.	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet (was obtained with iodine in mins.)	Brownish red (was obtained with iodine in mins.)	Light yellow (was obtained with iodine in mins.)	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	None	3·9	80	150	Not obtained	0·5
2	0·1 sistoamylase 1	1·7	150	240	,,	0·7
3	0·3 ,,	0·2	Without change after 4 hours			Not liquefied by 3 ml.
4	0·5 ,,	0·1	,,			,,
5	0·7 ,,	0·1	,,			,,
6	0·1 sistoamylase 2	0·7	Nearly without change after 4 hours			1·0
7	0·3 ,,	0·1	,,			Not liquefied by 3 ml.
8	0·5 ,,	0·1	Without change after 4 hours			,,
9	0·7 ,,	0·0	,,			,,

These results show that the plant sistoamylase also inactivates pancreatic amylase. 0·3 g. of the sistoamylase preparations inactivates 100 ml. of 0·25 % pancreatic amylase in 8 mins.

(b) The inactivation of pancreatic amylase by sistoamylase in presence of peptone.

(1) *Peptone an antisistoamylase substance to pancreatic amylase.* The effect of peptone on the sistoamylase-pancreatic amylase system was investigated in a similar manner, filtered solutions of 0·25 g. pancreatic amylase in 100 ml. water being used and shaken for 8 mins. after the additions had been made.

Table VI.

No. of solution	Additions	Saccharifying power ml. *N*/20 I	Dextrinising power: Violet (was obtained with iodine in mins.)	Dextrinising power: Brownish red (was obtained with iodine in mins.)	Dextrinising power: Light yellow (was obtained with iodine in mins.)	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	None	3·9	98	156	Not obtained	0·4
2	0·3 g. dried sistoamylase	0·1	Without change after 4 hours			Not liquefied by 3 ml.
3	„ +2 g. peptone	16·6	20	30	85	0·2
4	2 g. peptone	16·8	18	28	78	0·1

This table shows that only a very small quantity of free amylase is present in the solution of pancreatic amylase. Besides this, there is about four times as much amylase inactivated by a specific paralyser. The latter can be put out of action by peptone. We see further that the peptone acts also as anti-substance to added plant paralyser, just as in the case of the salivary amylase.

(2) *Peptone an eleutoamylase substance to pancreatic amylase.* In order to prove that peptone has the capacity of eluting the pancreatic amylase from the system pancreatic amylase + plant paralyser, the amylolytic activity of the following solutions was examined:

No. of solution																		
1	0·25 g. of pancreatic amylase + 100 ml. of water 5 mins. mechanically shaken and ... ... filtered																	
2	As above	Afterwards	+0·3 g. dried sistoamylase.				Shaken	8	mins. and then	+2 g. of peptone,				eluted	10	mins. and	„	
3	„	„	+0·3	„	„	„	8	„	„					30	„	„	„	
4	„	„	+0·3	„	„	„	8	„	„					60	„	„	„	
5	„	„	+1·0 g. undried		„	„	8	„	... ...							and	„	
6	„	„	„	„	„	„	8	„	+ 2g. of peptone,					eluted	10	mins. and	„	
7	„	„	„	„		„	15	„	„					10	„	„	„	
8	„	„	„	„	„	„	30	„	„					10	„	„	„	
9	„	„	„	„	„	„	60	„	„					10	„	„	„	
10	„	„	„	„	„	„	8	„	„					30	„	„	„	
11	„	„	„	„	„	„	15	„	„					30	„	„	„	
12	„	...	... ...		...	...	...	...	...	+2 g. of peptone,				...	...		„	

These experiments (Table VII) show that dried sistoamylase adsorbs the pancreatic amylase much more strongly than undried. We see also that the adsorption of the pancreatic amylase is the more complete, the longer the undried sistoamylase acts, so that it becomes more and more difficult to elute the amylase.

Table VII.

No. of solution	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet was obtained with iodine in mins.	Dextrinising power: Brownish red was obtained with iodine in mins.	Dextrinising power: Light yellow was obtained with iodine in mins.	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	3·9	98	156	Not obtained	0·4
2	0·1	Nearly without change after 4 hours			Not liquefied by 3 ml.
3	0·4	210	Not obtained		2·8
4	0·7	180	220	Not obtained	2·0
5	0·1	Nearly without change after 4 hours			Not liquefied by 3 ml.
6	11·1	28	41	106	0·1
7	9·0	35	48	135	0·1
8	7·0	45	60	192	0·15
9	6·3	52	73	216	0·2
10	12·0	24	46	94	0·1
11	8·6	36	53	192	0·15
12	16·8	18	28	78	0·1

Discussion.

Our researches on animal amylase give us not only an explanation of its relations to the natural plant paralyser, our sistoamylase substance, but also explain the manner in which different systems of animal amylase act under the influence of eleuto-substances.

We have ascertained that animal amylase, similarly to vegetable amylase, shows, depending on its origin and conditions of action, different mutual ratios of the three functions of amylolytic activity, the liquefying, dextrinising and saccharifying powers.

We see further that animal amylase under the influence of a plant paralyser is inactivated. This inactivation is of different grade depending on the sisto-amylase substance, on the time of its activity and on the origin of the amylase. It is to be noticed that dried sistoamylase substances have a greater power of adsorption than undried. The degree of adsorption increases with the time of action of the paralyser on the amylase.

The most interesting result of our investigation is that in salivary and pancreatic amylases substances inactivating the amylase are found. The result of this is that only a part of amylase is able to act, *e.g.* in a solution of salivary amylase only about 1/3 and in a solution of pancreatic amylase only about 1/4–1/5 of the total amylase is free. It can be deduced that natural paralysers of amylase, sistoamylase substances, occur along with the amylase in the animal, just as in the vegetable, organism. Peptone acts towards the plant paralyser in the same way as to the paralyser found in the solutions of animal amylase. Peptone added to the system plant sistoamylase + animal amylase prevents permit inactivation of the amylase, and also has the power of reactivating the amylase already inactivated by the sistoamylase substance originally present. The adsorption of animal amylase by an animal sisto-substance seems not to be so strong as the adsorption in the system plant sistoamylase + animal amylase. From the first system, especially in solutions of pancreatic amylase, the amylase is freed with more facility, and its amylolytic activity is restored. From the second system the amylase is freed with more difficulty, and in the system dried plant sistoamylase + animal amylase the amylase is so strongly adsorbed that it can only be eluted with difficulty by adding peptone. It is an interesting fact that peptone does not act on all the functions of the amylase equally. As

shown by our experiments, the saccharifying power is maximum on addition of 0·5 g. of peptone to 40 ml. of 5 % solution of salivary amylase. The dextrinising and liquefying powers show the best activity when 1–2 g. peptone are added to the same quantity of solution.

The question of the paralyser of amylase was treated for the first time by Wolff and Fernbach [1906], who introduced into the literature the term anti-amylase for the agent obtained by subcutaneous immunisation of rabbits to malt amylase. This phenomenon has been the subject of numerous researches by different workers. These investigations show that the anti-amylase acts as an inhibitor only on vegetable amylase. Abderhalden and Wertheimer [1922] not only question its paralysing action, but reject in general the existence of this anti-amylase. Kiselew [1928] distinguishes another kind of inactivation in the adsorption of amylase by vegetable plasma, coagulated by warming. Oparin and Kurssanow [1929] showed that in these cases the result was not due to the specific action of the plasma but to the inhibiting action on amylase caused by the presence of tannin.

The most interesting researches in this direction are those of Lesser [1919; 1921] and of Kerner and Lesser [1919], who proved that amylase of the liver is partly inactivated by adsorption, and that the retardation of the decomposition of glycogen may take place not only *in vivo*, but may also be reproduced experimentally by the addition of some adsorbent to the solution. The development of the details of this question is given by Przyłęcki *et al.* [1927]. Lesser showed, using adrenaline as stimulant, that the amylase of the liver can be freed, so that the active concentration of the ferment increases by elution of the inhibitor. Truszkowski [1928] finds that amylase can be inactivated by adsorption on lipoids to the extent of about 67 % of that present in the solution. The amylolytic action of the inactivated amylase can be restored almost quantitatively by the addition of an appropriate quantity of propyl or butyl alcohol.

The activating influence of peptone and of amino-acids is described by Effront [1900] and by Pozerski [1902]. Oppenheimer's [1927] opinion is that here we have to do with a change of p_H, though it is not excluded that these substances may have a real stimulating influence, as was proved by the researches of Sherman *et al.* [1921; 1922] and Rockwood [1924], who took the p_H into consideration. Oppenheimer supposes that this phenomenon can be explained on the ground that peptone protects the amylase from inactivation by rise of temperature.

According to our experiments the so-called phenomenon of activation of animal amylase is to be explained quite differently. We have shown that the whole of the amylase is not active, but only a portion of it. Very often a large portion of it is inactivated by adsorption by different accompanying substances, which are called by us sistoamylase substances. If peptone is added to a system of sistoamylase-amylase the action of the sistoamylase is excluded, the active concentration of amylase increases by elution, and in this case, we often find in the solution several times more free amylase, which according to its quantity will develop a correspondingly greater activity. The increase of amylolytic activity when different substances are added to the solution of animal amylase is nothing else than an elution of amylase from the system sistoamylase-amylase, in which a part of the amylase was adsorbed by the accompanying adsorbent. We are inclined to believe that various phenomena of the protection of amylase from inactivation, as for instance by moderate use of temperature, are in reality complete or incomplete hindrances of adsorption of the amylase by substances already present or formed during the treatment.

SUMMARY.

Experiments on salivary and pancreatic amylase lead us to the following results.

1. Plant sistoamylase inactivates not only plant but also animal amylase. Dried sistoamylase inactivates amylase more powerfully than undried.

2. In the place of origin of amylases as well as in their solutions natural paralysers, the so-called animal sistoamylases, are also found. The system sistoamylase-amylase is thus formed, in which the amylase is partly inactivated. Usually about 1/3 of the salivary and about 1/5 of the pancreatic amylase is not adsorbed.

3. In the system plant or animal sistoamylase-animal amylase, in which the amylase is inactivated, the amylase can be reactivated by the aid of eleutosubstances of which one of the best known by us is peptone. Certain forms of dried sistoamylase are of such adsorbing power that the amylase cannot be eluted by the use of peptone.

4. We explain the increase of amylolytic action of amylase on the addition of certain substances as an elution of amylase, which in this manner is rendered free to act.

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XLII. THE FORMATION OF SULPHATE FROM CYSTEINE AND METHIONINE BY TISSUES *IN VITRO*.

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(*Received January 8th, 1934.*)

In spite of the very large amount of work that has been done on the variations in the rate of excretion of sulphate by animals and men after the administration of various sulphur compounds, it seems that no one has shown that either an organ or a tissue preparation can make sulphate from an unoxidised sulphur compound *in vitro*. Rothera [1905] could find no sulphate when he incubated cystine with liver mince or when he perfused the liver with cystine, and it seems probable that several other workers have been similarly unsuccessful. The formation of sulphate from sulphuric esters by minced mammalian organs has been studied by Neuberg and Simon [1925], but this process, although perhaps connected with the type of oxidation which forms the subject of this paper, is obviously relatively simple.

The organ slice technique of Warburg [1923] is admirably suited to this work, for it causes little or no damage to the cells, and a series of experiments can be carried out under exactly comparable conditions, using parts of the same liver. By this means the troubles caused by the extreme individual variations between animals are avoided.

In this paper are described some preliminary experiments on the formation of sulphate by slices of kidney and liver. Later it is hoped that a larger group of substances will be investigated, as well as the probable intermediate stages in the oxidation and the extremely fragile enzyme systems by which these oxidations are carried out.

Experimental.

Rat tissue was used in all the experiments recorded; the animal was stunned and the required organs removed immediately and sliced as soon as possible. 30–80 mg. (dry weight) of slices were put in a vessel, of the type described by Krebs [1933], containing 8 ml. of bicarbonate Ringer solution. The latter was made up according to Krebs's directions [1932], except that magnesium chloride was used in place of magnesium sulphate. The vessels were filled with a gas mixture consisting of 95 % O_2 and 5 % CO_2 and shaken at 37°, generally for two hours. At the end of this time the flasks were taken out of the bath, rinsed on the outside with distilled water, dried round the joint with filter-paper and opened. The organ slices were removed with forceps, as much Ringer as possible was drained from them, and they were dried for 2–3 hours at 100° before being weighed. 2 ml. of 20 % trichloroacetic acid were added to the fluid in each flask, the mixture was poured into a 10 ml. pointed centrifuge-tube and the

protein precipitate centrifuged down. As a general rule the supernatant fluid was perfectly clear, but it sometimes contained small particles of tissue which floated because of entangled bubbles of CO_2. If the tube were shaken gently these could be centrifuged down.

Estimation of sulphate.

The method used for the estimation of sulphate is essentially that of Cuthbertson and Tompsett [1931]. In this the sulphate is precipitated in 70 % acetone with benzidine, the precipitate is washed thoroughly, and then the benzidine is estimated by diazotisation and coupling with thymol. Cuthbertson and Tompsett use a centrifuge throughout but very much more consistent results are obtained by using a sintered glass funnel (Schott 13f G4) of the type used by Pregl for the estimation of sulphur. The filter, mounted on a rubber bung, is placed inside a glass tube about 2 inches long and an inch wide, into the lower end of which the bung fits. This bung also carries a draining tube and an inlet tube for compressed air. The top of the wide glass tube can be closed firmly by another bung; fluid that is in the filter may then be forced through by opening the compressed air tube and closing the draining tube.

2 ml. of the centrifugate are added to 5 ml. of a 0·5 % solution of benzidine in acetone. The mixture is left in a stoppered tube for at least two hours on ice, it is then filtered by suction through the sintered glass funnel, and the tube is rinsed out on to the filter with acetone. The benzidine sulphate is washed by filling up the funnel (which holds 3 ml.) three times with acetone while it is connected with the pump. Care must be taken to wash the lip of the funnel during this process. The funnel is filled a fourth time, and the acetone is forced through by air pressure, the stem of the funnel being washed thoroughly with water during this last filtration. Two 3 ml. lots of boiling $N/3$ hydrochloric acid are used to transfer the benzidine sulphate to a test-tube; the fluid is forced through the filter by air pressure in each case. After sucking a little more $N/3$ HCl through the filter it is once more ready for use. The whole cycle of operations takes 6–10 minutes and the filter requires cleaning, by sucking nitric acid through it in the reverse direction, after about 10 estimations. At longer intervals it, like all the apparatus used in this work, is heated with cleaning mixture.

The benzidine is diazotised and coupled with thymol according to the directions of Cuthbertson and Tompsett except for the omission of the 15 % NaOH recommended by them[1]. The standard used was 0·3 ml. of a 0·053 % solution of benzidine hydrochloride in N HCl; this corresponds with 20γ of sulphur in the form of sulphate. The colorimeter cup containing the unknown solution is set at 20 on the scale, and the standard cup is adjusted to match; the standard scale now reads directly in thousandths of a mg. of sulphur in the form of sulphate in the 2 ml. sample of Ringer originally taken. On multiplying by five the figure that is recorded in the tables in this paper is obtained. All the estimations were done in duplicate, and the figure given is the mean of duplicates that did not differ by more than 1γ.

The cysteine used in this work was prepared by adding pyridine to an alcoholic solution of cysteine hydrochloride and washing the crystalline precipitate very thoroughly with alcohol. The glutathione, methionine and ergothioneine were prepared by the methods already published [Pirie, 1930; 1933, 1]. *S*-Ethylcysteine was prepared by the method of Clarke and Inouye [1931].

[1] I am very grateful to Miss E. Watchorn, of this laboratory, for pointing out the advantages of this modification.

Oxidation of cysteine and glutathione.

The results of a number of experiments on the oxidation of cysteine by liver slices are given in Table I. In each case the sliced liver, of which the dry weight is

Table I.

No. of rat	Wt. of cysteine in 8 ml. Ringer (mg.)	Dry wt. of liver (mg.)	Wt. of S found in the form of SO_4 (γ)
14	1·3	40	20
14	5·0	52	60
14	5·0	12	20
14	10·4	39	60
15	7	41	87 (72)
17	7	39	80
20	8	96	100
20	8 cystine	92	50
26	10	72	100
30	8	62	70
31	8	98	104 (100)
31	8 cystine	110	50
32	8	87	160
32	8	87	163

given, was suspended in 8 ml. of bicarbonate-Ringer containing the amount of cysteine specified. The flask was filled with the gas mixture and shaken for two hours at 37°. The figures in the fourth column are simply the amount of sulphur that was found in the flask in the form of sulphate at the end of the experiment; a series of blank experiments with cysteine alone, or with liver but no cysteine, or with liver slices that have been inactivated by heating to 60° or by prolonged freezing gave values ranging from 10 to 15γ. It is clear therefore that the sulphate concentration can be increased 5 or 10-fold by the oxidation of cysteine in this way. The reliability of the method used for the estimation of sulphate is also tested by the experiments done in nitrogen and by those in which different organs were used; these are described later.

The quantity of sulphate formed is too small to be estimated accurately by precipitation as barium sulphate followed by weighing. In a few cases, however, this has been attempted. A 4 ml. sample of the trichloroacetic acid centrifugate was used, and the weight of sulphur corresponding with the observed weight of barium sulphate was multiplied by 10/4; the figure so obtained is recorded in brackets in column 4 of certain of the tables. The two sets of figures are in sufficiently good agreement to make it certain that it is sulphate that is being estimated by the colorimetric method. It is necessary to emphasise this point, because the benzidine methods for the estimation of sulphate have often been criticised on account of their lack of specificity. Furthermore, some substances, cystine for example, are precipitated by the high concentrations of acetone used in the estimation. I find, however, that so long as sufficient trichloroacetic acid has been added in the first place, these precipitates can easily be washed free from adsorbed benzidine; they do not therefore interfere with the estimation. Small amounts of phosphate do not matter, but if it is present in large amounts sulphate estimations become impossible.

Table II contains the results of experiments in which cysteine is oxidised by kidney. Liver and kidney have much the same oxidising capacity, but the blood, testis, spleen, heart and lung of the rat seem to be quite inactive under these

Table II.

No. of rat	Wt. of cysteine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
16	8	26	55
20	8	42	90
27	7	48	100
28	7	63	100
30	8	36	70
31	8	34	50
31	8	38	60 (50)

conditions. The study of the kinetics of sulphate formation from cysteine is complicated by the fact that under the conditions of these experiments the cysteine is being rapidly oxidised to cystine; this separates as minute crystals if the concentration exceeds about 1 mg. per ml. Although cystine at lower concentrations than this stays in solution it cannot be directly oxidised to sulphate. If cystine is shaken with tissue slices, some sulphate is indeed formed, but this is probably due to a preliminary reduction to cysteine by the systems which Hopkins and Elliott [1931] and Mann [1932] have described. Two experiments of this type are recorded in Table I. The cystine, dissolved in the minimum amount of dilute hydrochloric acid, was added to Ringer solution containing a corresponding amount of NaOH. No separation of free cystine could be observed.

If a number of flasks all containing the same quantities of cysteine and liver are set up, and the amount of sulphate present is estimated after various intervals, it is found that no more sulphate is formed after the nitroprusside reaction has disappeared. With 7–8 mg. of cysteine and 60–80 mg. dry weight of liver this generally takes from 60–90 minutes, but the time is variable, although the relationship is constant. The following experiment is more definite. 12 mg. of cysteine and 140 mg. dry weight of liver were shaken for an hour at 37° in 16 ml. of Ringer. The flask was then opened, 8 ml. of the fluid were withdrawn for sulphate estimation, and half the slices were taken out and placed immediately into another flask containing 6 mg. of cysteine in 8 ml. of Ringer, *i.e.* the same concentration of cysteine that the first flask, which now contains only cystine, had contained initially. Both flasks were then closed, refilled with gas mixture and shaken for an hour at 37°. The sulphate contents of each flask and of the fluid withdrawn from the first flask after one hour were estimated. Within the limits of the method the sulphate content of a 2 ml. sample was the same in each case. Clearly therefore no sulphate was formed during the second hour in the flask from which all the cysteine had disappeared, but a part of the same group of slices was very well able to make sulphate when supplied with fresh cysteine. This conclusion, that disulphides are first reduced to mercaptans before undergoing oxidation to sulphate, has already been reached by Lewis *et al.* [1924] and others from feeding experiments on the intact animal. Furthermore, cysteine but not cystine can undergo the further stage of oxidation by the disulphide form of thiocarbamide which I have already described [Pirie, 1933, 2].

Hele and Pirie [1931] and Schelling [1932] have found that the intact animal can oxidise glutathione to sulphate even if it is given subcutaneously. The figures in Table III show that glutathione is also oxidised by tissue slices. It is clear that kidney has a much greater capacity to oxidise glutathione than liver. This fact makes it probable that the first step is the hydrolysis of the tripeptide into its constituent amino-acids, for it is well known that kidney undergoes

Table III.

No. of rat	Wt. of glutathione mg.	Dry wt. of tissue mg.	Wt. of S oxidised to SO_4 (γ)
19	24	60 liver	30
19	15	60 liver	20
19	54	48 liver	65
19	54	43 kidney	105
19	9	43 kidney	68
21	30	64 liver	45
21	30	45 kidney	75
21	15	52 kidney	75
21	30	22 kidney	57
25	30	69 liver	40
25	30	29 kidney	50
25	60	30 kidney	65
25	20	44 kidney	65
25	10	44 kidney	55
29	21	56 liver	45

autolysis more rapidly than most tissues, and it is the only tissue in which Bierich and Kalle [1928] were able to find free cysteine. Confirmatory evidence can be got by applying Sullivan's test to the fluid in which liver or kidney has been shaken with glutathione. The former gives no trace of cysteine colour, *i.e.* there is less than 0·03 mg. in a 2 ml. sample, whereas the latter contains about 0·7 mg. The most probable explanation of this would seem to be that the peptide-splitting enzymes of kidney make cysteine more rapidly than the cysteine-oxidising enzymes can destroy it, whereas in the case of liver this is not so. Needless to say the fluid in which kidney has lain for two hours without the addition of glutathione gives no detectable Sullivan reaction.

Oxidation of methionine.

Both liver and kidney can oxidise methionine to very much the same extent. In both cases, however, the rate of oxidation is very much smaller than the corresponding rate in the case of a similar concentration of cysteine. These points are illustrated in Tables IV and V. It is clear that methionine has to be present

Table IV.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Wt. of S found in the form of SO_4 (γ)
4	40	90	60
4	80	70	30
8	40	60	90
8	20	59	50
9	10	40	50
9	20	32	55
9	30	55	75
9	40	47	47
10	40	70	95
13	24	45	60
15	40	55	60
17	40	54	60
24	30	52	52
30	30	74	97
32	24	91	52
32	24	78	50

Table V.

No. of rat	Wt. of methionine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
8	40	31	60
10	30	41	85
10	50	35	75
10	80	35	80
30	30	30	62

in twice or three times the concentration that is necessary in the case of cysteine to get an equal rate of formation of sulphate. Individual variation between rats makes comparisons difficult, but it would seem from the figures for rats 4 and 9 in Table IV that there is quite a definite optimum concentration for sulphate production from methionine. Evidence of this sort could not be obtained in the case of cysteine on account of the formation of cystine.

I have found it impossible to get consistent results in the presence of toluene or other disinfectants. It is exceedingly unlikely that the sulphate formation is due to bacterial action, and the relative constancy of the sulphate production in different experiments makes it still less probable. The figures in Table VI show that the amount of sulphate formed during the third hour of an experiment is the same as that formed during the first and second hours. This fact entirely excludes the possibility of bacterial action.

Table VI.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Duration of experiment (hr.)	Wt. of S found in the form of SO_4 (γ)
12	30	87	0·5	20 (25)
12	30	57	1·0	36
12	30	52	2·0	60 (50)
12	30	49	3·0	85 (90)

Chase and Lewis [1933] have found in the urine of rats receiving *dl*-methionine a substance which gives a red colour with sodium nitroprusside in the presence of sodium cyanide. I have tested the fluid in which tissue slices have been shaken with methionine and find in all cases a faint but definite nitroprusside reaction. This reaction is given neither by fluid to which no methionine has been added nor by methionine solutions shaken without tissue. The possible significance of this will be discussed later.

Table VII.

No. of rat	Wt. of *S*-ethyl-cysteine (mg.)	Dry wt. of tissue (mg.)	Wt. of S found in the form of SO_4 (γ)
16	10	64 liver	15
16	40	76 liver	35
16	80	71 liver	57
16	40	32 kidney	37

In Table VII the results of an experiment with *S*-ethylcysteine are given. At a concentration at which cysteine would be very rapidly oxidised and methionine would be oxidised at an appreciable though not optimum rate this substance is hardly oxidised at all. It is only in relatively concentrated solutions that a definite formation of sulphate can be shown. The three substances cysteine, methionine and *S*-ethylcysteine do, however, form a series so far as *in vitro* oxidations are concerned which is very unlike their behaviour in the intact dog [Pirie, 1932]. There cysteine and methionine resembled one another while *S*-ethylcysteine was dissimilar. It is likely that this difference is due to

the fact that the tissue slice experiments are not complicated by the excretory mechanism, whereby an intact animal gets rid of foreign compounds into the urine.

Miscellaneous experiments.

Neither liver nor kidney can oxidise ergothioneine to sulphate under these conditions. This is somewhat unexpected since the sulphur atom in a thiol-glyoxaline is very much more readily oxidised to sulphate by chemical means than that in cysteine or methionine. Ergothioneine has been tested as a catalyst in the oxidation of the other sulphur compounds; here also no effect could be found. Thiocarbamide also was not oxidised by liver slices when present in low concentration, nor did it catalyse the oxidation of cysteine. It seems therefore that this oxidation cannot be directly compared with the oxidation by H_2O_2 [Pirie, 1933, 2]. The extract obtained by freezing and grinding liver and then centrifuging out the particles was also tested both as a substrate and as an activator; it was inactive. In this connection it should be recorded that the tissues of a rat from which food had been withheld for 24 hours had the usual oxidising power; rat 30 in Tables I, II, IV and V illustrates this.

Table VIII.

No. of rat	Wt. of substrate (mg.)	Dry wt. of liver (mg.)	Gas mixture	Wt. of S found in the form of SO_4 (γ)
17	None	53	95 % N_2 and 5 % CO_2	6 (20)
17	40 methionine	41		10
17	8 cysteine	49		15 (20)
17	30 methionine	46	95 % O_2 and 5 % CO_2	45
17	40 methionine	54		60
17	8 cysteine	39		80
17	30 methionine	53	Air	25
17	30 methionine	40	97·5 % O_2 and 2·5 % CO_2	45

Table VIII relates to the effect of different atmospheres on the oxidation by liver. The results call for little comment; as might be expected the oxidation does not proceed anaerobically. The oxidation is slow in air and there is no appreciable difference between the results obtained when a gas mixture containing 5 % CO_2 (p_H of Ringer 7·3) or one containing only 2·5 % CO_2 (p_H of Ringer 7·6) was used.

Little attempt has yet been made to investigate either the stability of this enzyme system or the effect of inhibitors upon it. Cooling to about $-5°$ generally inactivates the slices, and heating for 20 minutes at 60° does so with certainty, but short periods of freezing give erratic results. Finely ground tissue is inactive. The action of inhibitory substances is complicated by their effect on the autoxidation of the cysteine; a description of the effects observed will be published shortly.

Discussion.

Lawrie [1932] found that a rat, of the strain used in this laboratory, weighing about 240 g. excreted 10 mg. of sulphur in the form of sulphate per day when eating a synthetic diet. By feeding cystine the sulphate excretion could be raised to 20 mg. S per day, but at this level there was a definite rise in the neutral sulphur also. Now the liver and kidneys of a 240 g. rat weigh together about 12 g.; their dry weight is therefore 2·4 g., and this quantity of tissue will seldom be called on to oxidise more than 20 mg. of sulphur to sulphate in 24 hours. 50 mg. dry weight of tissue should on this basis oxidise 35γ of sulphur to sulphate in 2 hours. The figures in Tables I and II show that tissue slices can make sulphate from cysteine at a rate considerably in excess of this if the cysteine

concentration is raised to unphysiologically high levels. Since, however, the sulphate-forming mechanism, when working *in vitro*, has to compete with the autoxidation processes this agreement is fairly satisfactory. The figures in Tables IV and V are more important, for methionine is the principal sulphur-containing constituent of most diets. When we consider the very high concentrations of methionine that are being used and also the fact that the action is proceeding in 95 % oxygen rather than in air, it is clear that there is little quantitative relation between the rates at which rat organs can oxidise methionine *in vitro* and *in vivo*.

It would be premature to put forward a theory of the mechanism of this oxidation with any degree of confidence. As a working hypothesis, however, the following scheme is suggested:

$$\begin{matrix} \text{R.SS.R} \\ \text{or} \\ \text{2R.SCH}_3 \end{matrix} \rightarrow \text{2R.SH} \rightarrow \text{2R.SOH} \rightarrow \text{R.SH} + \text{R.SO}_2\text{H} \rightarrow \text{H}_2\text{SO}_3 \rightarrow \text{H}_2\text{SO}_4$$

Of these processes the first and second are probably enzymic, the third proceeds spontaneously at body p_H [Pirie, 1933, 2], the fourth is enzymic and the fifth need not necessarily be so. The relatively slow rate at which sulphate is formed from methionine, taken in conjunction with the occurrence of disulphides in the fluid in which methionine is being oxidised, is at least consistent with the theory that this substance has to be demethylated before it can be oxidised.

Summary.

The oxidation of cysteine and methionine to sulphate by slices of rat liver and kidney has been studied.

Cystine can only be oxidised after undergoing reduction and glutathione only after hydrolysis.

Ethylcysteine is oxidised slowly and ergothioneine not at all.

A modification in the technique of micro-sulphate estimation is described.

I am very grateful to Sir F. G. Hopkins for his interest in this work and to Dr H. A. Krebs for his advice on many points.

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XLIII. THE CONSTITUTION OF COCCERIN.

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(*Received January 8th, 1934.*)

Introduction[1].

Liebermann [1885] showed that boiling benzene extracted an alcohol-insoluble wax which he called coccerin from the cochineal insect (*Coccus cacti*). By saponification with strong alcoholic potash and separation of the acids as calcium soaps he obtained an alcohol, cocceryl alcohol, which melted at 101–104°, and an acid, coccerinic acid, which melted at 92–93°. Two years later Liebermann and Bergami [1887] investigated the structure of these two compounds. Analysis of cocceryl alcohol suggested that it was a C_{30}-alcohol with two oxygen atoms. Acetylation with acetic anhydride in a sealed tube at 170° gave a product, m.p. 48–50°, apparently a diacetate; similar evidence was obtained on benzoylation, and it was concluded that cocceryl alcohol was a dihydric alcohol $C_{30}H_{60}(OH)_2$. On oxidation with chromic acid it gave an acid m.p. 59–60° which was identified as pentadecanoic acid. Analysis of the coccerinic acid, and of its salts, suggested that it was a hydroxy-acid $C_{31}H_{62}O_3$, although no evidence for the hydroxyl group was obtained by acetylation or benzoylation. This acid on oxidation was also stated to give pentadecanoic acid, which was identified as its methyl ester, m.p. 66–68°. As coccerin gave by saponification two parts of acid to one of alcohol it was considered to be $C_{30}H_{60}(C_{31}H_{62}O_3)_2$, in keeping with the dihydric nature of the alcohol.

The wax has been more recently examined by Becker [1931], who recrystallised it many times from benzene and chloroform, thereby raising the m.p. to 104–105°. By saponification with $N/2$ alcoholic potash and separation of the calcium soaps he obtained approximately equal amounts of alcohol, m.p. 102°, and of acid, m.p. 94–95°. His analysis suggested that the alcohol was $C_{32}H_{66}O_2$, and on acetylation in the usual way with acetic anhydride and fused sodium acetate it gave a monoacetate, m.p. 78–79°. Treatment in a sealed tube with acetic anhydride at 170° gave a mixture from which only the monoacetate could be recovered. His analysis of coccerinic acid agreed better with the formula

[1] The melting-points below 110° (except those of oximes) recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

The nomenclature used in this paper is based on the following system, which has recently been made official (permissive) by the International Union of Chemistry [*cf. J. Chem. Soc.* (1931), 1610].

$R.CH_3$	$R.CH_2OH$	$R.CHO$	$R.CO_2H$	$R.COCl$	$R.CONH_2$	$R.CH_2NH_2$
-ane	-anol	-anal	-anoic acid	-anoyl chloride	-anoamide	-anamine

The number will be placed immediately before the suffix to which it refers, and the C of the CO_2H group will be numbered 1. The system is more logical than the one previously used in publications from this laboratory and will be adopted in future. A. C. C.

$C_{32}H_{64}O_3$, and accepting Liebermann and Bergami's isolation of pentadecanoic acid, he concluded that cocceryl alcohol and coccerinic acid had corresponding structures. Coccerin therefore had the same biological origin as the plant waxes, and its components were formed by a Cannizzaro reaction from two molecules of an aldehyde built up by repeated aldol condensation.

In connection with the research which is being carried out in this laboratory into the metabolism of waxes this cocceryl alcohol was of interest. Since pentadecanoic acid is now known to melt at 54° and its methyl ester at about 19° we were not prepared to accept all Liebermann's conclusions; in particular Becker's failure to obtain a diacetate suggested to us that the second oxygen atom might be ketonic. Clemmensen reduction might therefore provide us with *n*-dotriacontanol, an alcohol which would be extremely useful in connection with the X-ray analytical work which we are undertaking in conjunction with Dr Piper.

Our first preparation of the wax was made from 15 kg. of cochineal by Liebermann's method. By saponifying in strong benzene-alcoholic potash and separating the acids as their calcium soaps there were obtained approximately equal amounts of alcohol and acid, confirming the observations of Becker. The alcohol after many recrystallisations from benzene and alcohol melted at 99–100°. A typical example of one out of many analyses confirmed the data of both Liebermann and Becker, *viz.* C, 79·5 % and H, 13·2 %. On acetylation with acetic anhydride in the presence of fused sodium acetate we obtained a monoacetate, again confirming Becker's results. X-ray analysis gave a good photograph, 23 orders measuring 90·4Å. At the time we could not be certain of the chain length, but we were able to deduce two facts: (1) if the chain length were about 32 carbon atoms, then the doubled spacing showed that the alcohol group was attached to the last or penultimate carbon atom; (2) the intensity distribution showed that another polar group was attached at some point in the carbon chain.

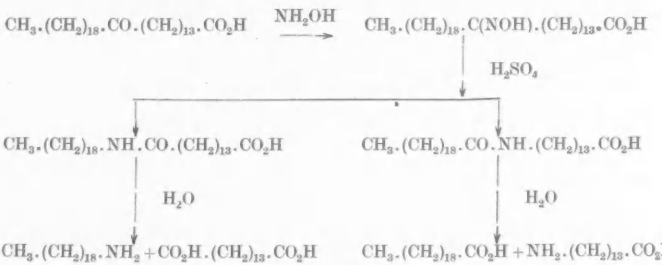
Treatment with hydroxylamine readily gave an oxime, M.P. 77·5–79°, confirming our surmise that the second oxygen atom was ketonic. Clemmensen reduction gave a monohydric alcohol, M.P. 89·7°, and this on reduction *via* the iodide a paraffin. The melting-point and transition temperatures of the latter showed that it was slightly impure *n*-tetratriacontane, and this was confirmed by X-ray analysis, which gave both the A and C spacings, and we know that the presence of the C spacing is specific [Piper *et al.*, 1931]. X-ray analysis of the monohydric alcohol gave a fair picture, 11 orders measuring 90·2Å. At this stage in our research the melting-points and crystal spacings for the *n*-fatty acids and primary alcohols with more than 26 carbon atoms were not known, but by extrapolating the data obtained by Malkin [1930] from the primary alcohols with 12 to 25 carbon atoms this spacing gave a reasonably good agreement for the primary alcohol with 34 carbon atoms. Cocceryl alcohol was therefore a keto-*n*-tetratriacontanol, which requires C, 80·2 % and H, 13·5 %. These values were appreciably higher than we and the above-mentioned authors had obtained, and it was clear that some oxygen-rich impurity was present, because a small amount of unsaponified wax would not lower the carbon content. Repeated attempts to purify the alcohol and its acetate with various solvents were not very successful.

Another preparation of wax from 15 kg. of cochineal was obtained, and this was submitted to repeated extraction with large volumes of benzene-alcohol (1:5). After the tenth extraction the residue melted sharply at 106° and its weight was only about one-third that of the original wax. This was saponified first

with benzene-alcoholic potash and then with sodium ethoxide, the acids in both cases being converted into the calcium soaps. The cocceryl alcohol thus obtained melted sharply at 100–100·2°, and on analysis gave, within experimental error, the correct figures, *viz.* C, 80·0 %, H, 13·5 %. Although this procedure undoubtedly removed the impurity it was wasteful, and as we were desirous of collecting a large quantity of both cocceryl alcohol and the acids for further analysis we repeated it with another batch of wax (from 30 kg. of cochineal) at the same time paying attention to the products extracted by the benzene-alcohol, which were finally collected into two fractions. Full details are given in the experimental section. The results show clearly that extraction with hot benzene-alcohol does not *per se* effect very much purification. As will be discussed later, the so-called coccerinic acid of Liebermann is a mixture of *n*-fatty acids and a ketonic acid, so that coccerin is a mixture of true waxes whose solubility in hot benzene-alcohol will naturally depend on the chain-length of the particular acid component. The impurity present is undoubtedly stable to saponification with alcoholic potash, but is converted into water-soluble products by saponification with sodium ethoxide, and it is the omission of this essential step which has led to the misleading conclusions drawn from earlier work.

The purified cocceryl alcohol on Clemmensen reduction gave a monohydric alcohol, M.P. 92·2°, which was shown to be *n*-tetratriacontanol by (1) reduction *via* the iodide to *n*-tetratriacontane, M.P. 72·6–72·8°, and (2) oxidation to *n*-tetratriacontanoic acid, M.P. 98·3–98·5°; the purity of all three products was confirmed by X-ray analysis.

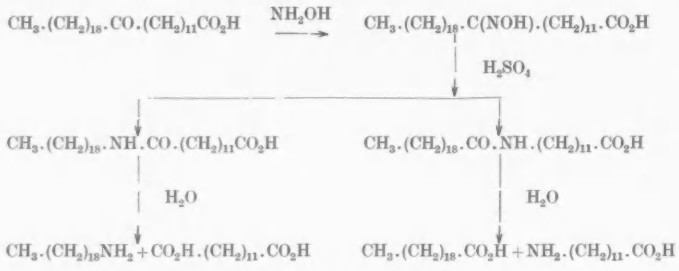
Oxidation of the keto-alcohol with chromium trioxide in glacial acetic acid gave the corresponding ketonic acid in good yield. This was treated with hydroxylamine and the resulting oxime converted into the corresponding amides by heating with concentrated sulphuric acid. These amides were hydrolysed by heating with hydrochloric acid and the products thus obtained were identified as *n*-nonadecanamine, *n*-eicosanoic acid, *n*-tridecane-(1:13)-dicarboxylic acid and 14-aminomyristic acid. From this it follows that the ketonic acid is 15-keto-*n*-tetratriacontanoic acid and that the above products are formed as follows:



Cocceryl alcohol is therefore 15-keto-*n*-tetratriacontanol.

The acids obtained from the three fractions of the wax, the so-called coccerinic acid of Liebermann, showed variations in melting-point suggesting that they were mixtures similar to those met with as components of plant waxes. Analysis for carbon and hydrogen however again showed the presence of excess oxygen above that required for appropriate mixtures of *n*-fatty acids. Each fraction was unaffected by acetylation, showing that the acids were not hydroxy-acids,

as had been suggested by Liebermann. But treatment with hydroxylamine gave a product freely soluble in cold acetone which could be readily separated from an insoluble residue. This soluble product on purification melted in each case at 56–57°, and analysis showed that it was a ketoxime. Hydrolysis regenerated the ketonic acid, which melted at 104·5–105°. This was shown to be a keto-*n*-dotriacontanoic acid by Clemmensen reduction to *n*-dotriacontanoic acid. The position of the ketonic group was determined as before by heating the ketoxime with sulphuric acid to effect a Beckmann transformation. The resulting amides were hydrolysed with hydrochloric acid, and the products thus obtained were identified as *n*-nonadecanamine, *n*-eicosanoic acid, brassylic acid and 12-aminolauric acid. From this it follows that the ketonic acid is 13-keto-*n*-dotriacontanoic acid, and that the above products are formed as follows:



The constitution has been confirmed by synthesis.

The fraction of the acids insoluble in cold acetone was again treated with hydroxylamine to remove any residual ketonic acid and the product finally obtained recrystallised twice from acetone at 37°. This melted at 93·6° and was shown to be *n*-triacontanoic acid.

It will be clear from the above results that the "coccerinic acid" of Liebermann was a mixture, and as correctly the name should be applied to the acid corresponding to cocceryl alcohol, *i.e.* 15-keto-*n*-tetratriacontanoic acid, which has not yet been found in natural products, we recommend that it be abandoned.

This elucidation of the structure of coccerin is of great interest from the point of view of wax metabolism, for not only do the ketonic alcohol and ketonic acid provide a clear case of β-oxidation, but they both contain the group $CH_3.(CH_2)_{18}.CO$, which corresponds with the group $CH_3.(CH_2)_{18}.CHOH$ present in *n*-nonacosan-10-ol, a constituent of apple cuticle wax [Chibnall *et al.*, 1931]. Further discussion is reserved for a later paper.

The difficulty which we experienced in the early stages of this research in purifying coccerin and its constituent alcohol and acids has forcibly drawn our attention to two steps in the procedure which we now consider to be essential. In the first place the initial saponification of the wax with alcoholic potash should be followed by saponification with sodium ethoxide. This is a usual precaution if esters of sterols are known to be present, but it is necessary in the present case in order to remove more effectively impurities which are stable to boiling alcoholic potash. In the second place the alcohol and acid liberated by saponification cannot be effectively separated unless the acid is converted into the very insoluble calcium soap. Liebermann adopted this procedure, as also have certain more recent workers, but others have omitted it probably because it has not been generally realised that the potassium or sodium soaps of the

fatty acids with carbon chains longer than 26 become increasingly soluble in hot acetone or hot benzene with increase of chain length. For these two reasons we consider that most, if not all, of the previous work on the constitution of insect waxes is in need of reinvestigation, and it is to be regretted that in most cases the waxes themselves are so difficult to obtain even in small amounts.

This applies especially to the wax of *Pemphigus xylostei*, which has been recently investigated by Schulz and Becker [1931]. The insects were collected over a period of many years and gave 1·9 g. of wax. This was repeatedly crystallised from chloroform-alcohol, and the sharp melting-point, 108–108·5°, to which they call attention, suggests that extraneous material had been successfully removed. The wax was then saponified with benzene-alcoholic potash, the alkali removed by washing with water and the alcohol (pemphigus alcohol) separated from the acid (pemphigus acid) by extraction with hot benzene. The pemphigus alcohol was recrystallised many times from hot alcohol, but the indefinite melting-point (100–105°) shows clearly that the material was still contaminated with a small amount of potassium soap. Carbon and hydrogen analysis of the alcohol and of its acetate (M.P. 75–77°) suggested that it was a C_{34} dihydric alcohol, the higher homologue of cocceryl alcohol. Accepting Liebermann's isolation of pentadecanoic acid after oxidation of the latter they suggest that pemphigus alcohol is 18-hydroxytetratriacontanol. We do not agree with these conclusions. Their analysis of the alcohol (C, 80·0, 80·21; H, 13·46, 13·50°) agrees much better with that of a ketotetratriacontanol (C, 80·2; H, 13·5 %) than with that of a hydroxytetratriacontanol (C, 79·9; H, 13·8 %). The melting-point (75–77°) of their acetate suggests to us the presence of only one acetyl group (*cf.* the acetate of cocceryl alcohol, M.P. 80·9–81·3°); they infer that it is a diacetate from the result of a single analysis for carbon and hydrogen on a very small amount of material. To obtain collateral evidence we have ourselves reduced cocceryl alcohol to 15-hydroxy-*n*-tetratriacontanol, and as we expected, the diacetate has a much lower melting-point (50–51°). Pending reinvestigation of this wax therefore we prefer to consider that pemphigus alcohol is a ketotetratriacontanol, and that in all probability it is identical with cocceryl alcohol. The melting-point of pemphigus acid (101–102°) is too low for a hydroxy- (or keto-) acid with 34 carbon atoms as they suggest, and it is undoubtedly a mixture similar to "coccerinic acid" but containing a small proportion of *n*-fatty acid, so that the melting-point of the pemphigus wax (108–108·5°) should be higher than that of coccerin (106–106·5°).

Experimental.

Preparation of the wax. 30 kg. of cochineal were extracted with boiling benzene by British Drug Houses, Ltd. The resulting extract, from which the main bulk of the solvent had already been removed before it was delivered to the laboratory, was boiled for 15 minutes with an equal volume (5100 ml.) of benzene and the mixture allowed to stand until cold. The product which separated was filtered off and recrystallised from 3 litres of boiling benzene. The weight of crude wax, dried in an air-oven at 90°, was 149 g., and the M.P. indefinite 99–102°.

Separation of the wax into three fractions. The crude wax was first boiled with 2 litres of 95 % alcohol for 6 hours and the mixture filtered hot. The residue melted at 103–104° and the material (2·5 g.) recovered from the solvent at 98–98·5°. The residue was then extracted seven times successively for 1 hour with 2 litres of boiling benzene-alcohol (1 : 4), the mixtures being filtered hot through a conical filter under slight suction. The residue melted at 105–106°, while the material recovered from the last hot filtrate melted at 100–101°. The total amount of material recovered from these first eight extractions (Fraction 1), weighed 35 g. Seven further extractions with similar amounts of benzene-alcohol were then made. The material recovered from these extracts (Fraction 2) weighed 34·9 g. and melted at 103–103·5°. The final residue (Fraction 3) weighed 62·0 g. and melted at 106–106·5°. A further 9·7 g. of crude wax were recovered from the numerous filter-papers used in the above separation and were neglected.

Saponification of the wax. The treatment of Fraction 3 given below applies equally to Fractions 1 and 2. The wax (62 g.) was dissolved in 1800 ml. of hot benzene, 1200 ml. of 10 % alcoholic potash were added, and the mixture was boiled under reflux for 12 hours. At the end of that period 3 litres of 95 % alcohol containing 160 g. of calcium chloride were added and the boiling continued for another 2½ hours. The mixture was then filtered hot, using a battery of hot-water-jacketed conical filters. The residual calcium soaps were extracted three times successively with 3 litres of boiling alcohol and then with the same volume of boiling acetone. The filtrates were cooled, and the crude cocceryl alcohol which separated was collected. This material (41 g.) was treated five times successively with boiling acetone and filtered hot. The filtrates on cooling gave 24 g. of crude alcohol. The residue insoluble in the hot acetone (12 g.) contained calcium soaps, which in the previous stage must have been rendered soluble in hot alcohol by the excess of potash present, a little unsaponified wax and much unidentified material. The crude alcohol and residue were therefore collected, dissolved in 1200 ml. of hot benzene and boiled under reflux for 3½ hours with sodium ethoxide (20 g. of sodium in 480 ml. of alcohol). 30 g. of calcium chloride in 250 ml. of 95 % alcohol were then added, and the boiling was continued for 2 hours. The mixture was filtered hot, and the insoluble soaps were extracted three times successively with 1 litre of boiling 95 % alcohol and finally with 1 litre of boiling acetone. The combined filtrates on cooling gave 26·5 g. of crude cocceryl alcohol, M.P. 99–100°. This was digested with 3½ litres of boiling acetone and filtered hot. The filtrate on cooling gave 15 g. of alcohol, M.P. 99·6–99·8°. The residue was treated three times successively in the same way, giving a further 4·7 g. of alcohol, M.P. 100·2–100·5°. The ultimate residue of soap weighed 0·8 g., and the acetone mother-liquors on evaporation gave 4 g. of crude alcohol which was neglected.

The calcium soaps from the first saponification were boiled under reflux with 2 litres of glacial acetic acid until the solution was clear and the mixture poured into an excess of cold water. The precipitated acids were collected, the treatment with glacial acetic acid repeated and the product crystallised from acetone. The material thus obtained (25·7 g.) was digested for some time with 3 litres of boiling acetone and filtered hot. On cooling, the filtrate gave 14·1 g. of fatty acid, M.P. 98·2–99°. Three further extractions of the residue gave in all 6·5 g. of fatty acid, M.P. 104–105°. The ultimate residue was neglected, as also were the 3 g. of crude acid recovered from the acetone mother-liquors on evaporation. The gross yield of acids recovered was 23·6 g. and of alcohol 23·7 g., showing a loss of 14·7 g.

Fraction 2 on similar treatment gave 13·1 g. of crude acids and 11·0 g. of crude alcohol, showing a loss of 10·8 g. The crude acids on digestion with hot acetone gave 11·6 g. of material, M.P. 98·0–98·4°, and the crude alcohol 9 g. of material, M.P. 99–100°. Fraction 1 in the same way gave 12·5 g. of crude acid and 11·0 g. of crude alcohol, showing a loss of 11·5 g. The acetone treatment gave 11 g. of acid, M.P. 89–90°, and 9 g. of alcohol, M.P. 100–101°. In all three fractions the major part of the loss occurred during the saponification with sodium ethoxide.

Fractionation of the fatty acids. The two samples of acid from Fraction 1, and the samples from Fractions 2 and 3 were separately treated with hydroxylamine in the following way. The acid (14·1 g.) in 800 ml. of alcohol was boiled under reflux for 8 hours with 6 g. of hydroxylamine hydrochloride and 9 g. of potassium hydroxide. The mixture was then poured into 6 litres of dilute sulphuric acid, the separated material filtered off and washed thoroughly with

water. It was then taken up in 1300 ml. of warm acetone; 300 ml. of ether were stirred in, and the mixture was allowed to stand for some hours at room temperature. 4·35 g. of *n*-fatty acid crystallised out and were filtered off. The other three samples gave in the same way 0·93, 6·35 and 1·8 g. of crystalline *n*-fatty acid, which were further treated as described later.

The acetone-ether mother-liquors were reduced to one-eighth of their original volume and cooled in the ice-chest. The material which separated was filtered off and dissolved in 1 litre of hot acetone. On cooling to room temperature a first crop of crude oxime weighing 6·4 g. was obtained. The mother-liquor was concentrated to 200 ml. and cooled in the ice-chest. A second crop of crude oxime was thus obtained. The latter was repeatedly extracted at room temperature with changes of cold acetone (shaking and centrifuging) until only a small residue remained undissolved. On evaporation of the solvent the oxime was recovered, and the cold extraction was repeated. The oxime (12·75 g.) then crystallised out in rosettes of white needles.

Constitution of the ketonic acid.

Preparation of the ketonic acid from the ketoxime. The ketoxime melted at 56–57°. (Found: C, 75·5; H, 12·5; N, 2·4 %. $C_{32}H_{62}O_2.NOH$ requires C, 75·4; H, 12·5; N, 2·7 %.) The regeneration of the ketonic acid from the ketoxime was found to be unexpectedly difficult, owing presumably to a small amount of amide formation. The following procedure was tedious and wasteful, and we are hoping that further research will enable us to increase the yield materially. 9·8 g. of ketoxime were warmed on the water-bath with 100 ml. of formaldehyde (40 %), and then 100 ml. of concentrated hydrochloric acid were added with stirring [Lapworth, 1907]. Separation of solid material rapidly took place and appeared to be complete in about 2 minutes. After remaining on the bath for about 15 minutes the mixture was poured into water, filtered and the residue washed with water and then crystallised from acetone. The material, which was recovered in quantitative yield, melted at 99·5–100°. After a prolonged fractionation from acetone at 37° there were finally obtained 1·9 g. of the ketonic acid, M.P. 104·5–105°. (Found: C, 77·9; H, 12·7 %. $C_{32}H_{62}O_3$ requires: C, 77·7; H, 12·6 %.) The lowest-melting fraction obtained melted at 96·4–96·7° and contained about 0·2 % of N (Dumas).

Conversion of the oxime of the ketonic acid into the amides. The oxime (4·4 g.) was heated on the water-bath with concentrated sulphuric acid (25 ml.) for 2½ hours. The brown solution was cooled and slowly poured on to crushed ice, and the precipitated amides were collected and crystallised first from 250 ml. of glacial acetic acid and then from an equal volume of acetone. The product obtained was a faintly pink crystalline powder melting at 102°.

Hydrolysis of the above amides. 2·5 g. of the amides were heated with 12 ml. of concentrated hydrochloric acid in a sealed tube at 180° for 4 hours. The resulting dark brown products were mixed with 100 ml. of water and distilled in a current of steam. No volatile acids were separated. The aqueous mixture was therefore filtered and the insoluble material taken up in hot alcohol and filtered to remove charred products. The filtrate was concentrated to 50 ml. and 2 volumes of ether were added. On standing overnight a mass of thin white crystalline flakes separated, which were filtered off (Fraction A; wt. 0·29 g.). The filtrate was concentrated to 10 ml.; 20 ml. of hot benzene were added and the mixture allowed to stand overnight. In the morning a further 30 ml. of benzene were added with stirring and the white solid which separated was filtered off (Fraction B; wt. 0·38 g.). The mother-liquor was taken to dryness and the

residue dissolved in 60 ml. of hot alcohol. The solution was made alkaline to phenolphthalein with alcoholic sodium hydroxide and allowed to stand overnight. A white powder separated and was filtered off (Fraction C; 0·45 g.). The mother-liquor on evaporation gave Fraction D.

Fraction A. This was crystallised from 40 ml. of hot benzene, and gave 0·26 g. of material which was then repeatedly extracted with ether. The insoluble residue was dissolved in 20 ml. of alcohol and 2 volumes of ether were added. The mass of thin colourless plates which separated overnight was filtered off and washed with ether. The M.P. was indefinite and analysis suggested that the substance was *n*-nonadecanamine hydrochloride. (Found: C, 71·3; H, 13·3; N, 4·2 %. $C_{19}H_{41}N.HCl$ requires C, 71·3; H, 13·2; N, 4·4 %.) For confirmation this amine, which has not hitherto been prepared, was synthesised from pure *n*-eicosanoic acid by the method of Jeffreys [1899]. The synthetic amine and the substance from Fraction A both gave benzoates crystallising from alcohol in fine needles melting sharply at 88° either alone or when mixed. Samples of *n*-octadecanamine hydrochloride and *n*-eicosanamine hydrochloride gave benzoates melting at 88·2° and 91·5° respectively, which were depressed when mixed with the benzoate from Fraction A.

Fraction B. This was extracted several times with boiling benzene and the insoluble residue crystallised from 20 ml. of 2*N* hydrochloric acid. A first small crop of crystals separated in a few hours and was filtered off. These melted at 145° and were found impossible to purify. The filtrate on long standing gave a second crop of material in the form of fine colourless lamellae. These melted at 163°, alone and when mixed with a sample of authentic 12-aminolauric acid hydrochloride prepared by the late Dr Le Sueur. (Found: (micro-Dumas) N, 5·6%. $C_{12}H_{25}O_2N.HCl$ requires: N, 5·6%.) The micro-Kjeldahl method gave a low result, possibly owing to ring formation. The free amino-acid was obtained by dissolving the hydrochloride (18 mg.) in the calculated volume of dilute sodium hydroxide solution. The white crystalline mass which separated on cooling was collected, washed with cold water and dissolved in boiling water (10 ml.) from which it crystallised on cooling in large thin plates. The M.P. was 183° (uncorr.), unchanged when mixed with a sample of authentic 12-aminolauric acid prepared by the late Dr Le Sueur (M.P. 183°). (Found: (micro-Dumas) N, 6·5 %. $C_{12}H_{25}O_2N$ requires: N, 6·5 %.)

Fraction C. This was dissolved in 30 ml. of glacial acetic acid to decompose the soaps and poured into water. The solid which separated was filtered off and crystallised from ice-cold acetone. Three crops were obtained melting at 66°, 72·5° and 70° respectively. The first of these was extracted several times with boiling light petroleum (B.P. < 40°) and the extracts were concentrated to 10 ml. The crystalline material which separated melted at 73°. This and the second and third crops mentioned above were collected (0·15 g.) and recrystallised twice from 10 ml. of 90 % alcohol. The final product melted at 74–74·2°, and analysis showed that it was *n*-eicosanoic acid. (Found: C, 77·2; H, 12·9 %; mol. wt. by titration 313. $C_{20}H_{40}O_2$ requires: C, 76·9; H, 12·9 %; mol. wt. 312.) A mixed M.P. of 74·4–74·8° was obtained with a sample of synthetic *n*-eicosanoic acid, M.P. 75·0–75·2°, which had been purified by fractional distillation of the ethyl ester. In a mixed melt with synthetic *n*-heneicosanoic acid, M.P. 74·2–74·6° the M.P. was depressed to 70·5°.

Fraction D. This was treated with ether and allowed to stand overnight. The solid which separated was filtered off and the ethereal filtrate extracted with water. The solid was dissolved in this aqueous extract, which was then acidified and again extracted with ether. The ethereal solution yielded 0·23 g.

of solid, which was extracted repeatedly with small quantities of light petroleum (B.P. < 40°). The residue was recrystallised twice from benzene, then from acetone (charcoal) and again from benzene. The product (0·09 g., M.P. 108°) was dissolved in 150 ml. of boiling water, which was filtered hot. Fine white needles separated on cooling (M.P. 111·5°). These were collected, dissolved in 30 ml. of boiling absolute alcohol and titrated hot to phenolphthalein with alcoholic sodium hydroxide. After standing some days small plates of the sodium soap crystallised out. These were filtered off and dissolved in 50 ml. of boiling dilute hydrochloric acid. The crystals which separated on cooling were shown to be brassylic acid. (Found: C, 64·3; H, 10·0 %; mol. wt. by titration (dibasic acid) 250. $C_{13}H_{24}O_4$ requires: C, 63·9; H, 9·9 %; mol. wt. 244.) The M.P. was 113°, unchanged when mixed with authentic brassylic acid (M.P. 113·5°).

*Synthesis of 13-keto-*n*-dotriacontanoic acid.* The method of Robinson [1930 and unpublished data] was used. Ethyl 11-bromoundecanoate was condensed with ethyl sodioacetoacetate to give ethyl 2-acetylbrassylate. Ethyl sodio-2-acetylbrassylate (from 4 g. of the ester) in anhydrous ether was added slowly to *n*-eicosanoyl chloride (from 2·8 g. of the acid) in anhydrous ether and the mixture allowed to stand overnight. The next morning it was boiled to complete the reaction, the ether removed and the condensation product extracted with 50 ml. of alcohol. 3 litres of 3 % alcoholic (95 %) sodium hydroxide were then added, and the mixture shaken for 10 hours on two successive days. Half the alcohol was then removed by evaporation and the remainder boiled under reflux for 1 hour. After cooling in the ice-chest the precipitated soaps were filtered off, decomposed by boiling with aqueous hydrochloric acid and the free acids crystallised first from acetone and then from glacial acetic acid. The yield of crude acid was 1·1 g., M.P. 99·5–100·5°. To remove residual *n*-eicosanoic acid the material was recrystallised three times successively from acetone at 37°. The melting-point was 104·5–105°, unchanged when mixed with the cochineal acid. (Found: (Schoeller) C, 77·7; H, 12·6 %. $C_{32}H_{62}O_3$ requires: C, 77·7; H, 12·6 %.) The oxime was prepared in the usual way and crystallised repeatedly from acetone. The melting-point was 58–59°, and 57–58° when mixed with the oxime of the cochineal acid, which melted at 56–57°.

Constitution of the n*-fatty acids.*

The four samples of *n*-fatty acids obtained during the fractional crystallisation of the ketoxime were collected and 12 g. again treated with hydroxylamine as before. 1·5 g. of crude oxime and 9 g. of *n*-fatty acid, M.P. 92·8–93°, were obtained. The *n*-fatty acid was recrystallised twice from acetone at 37°, when the melting-point was raised to 93·4–93·6°, unchanged on further treatment. It was *n*-triacontanoic acid. (Found: C, 79·5; H, 13·3 %; mol. wt. by titration 454, 453. $C_{30}H_{60}O_2$ requires: C, 79·6; H, 13·4 %; mol. wt. 452·5.) The mixed melt with pure *n*-triacontanoic acid (M.P. 94°) was 93·6–93·8°. The X-ray photograph (Series B, 271) gave 32 orders in the B form measuring 71·1 Å. as against 71·4 Å. standard. There was a trace of the C spacing in the 3rd order showing that the acid probably contained less than 1 % either of higher *n*-fatty acids or of the keto-acid which had escaped oximation. This is the first recorded isolation from natural sources of *n*-triacontanoic acid as distinct from "melissic acid," which melts at 90–91° and has been considered by various workers to be a normal or *iso*-fatty acid with either 30 or 31 carbon atoms. We shall deal with the constitution of this acid in a later paper.

Constitution of cocceryl alcohol.

The samples of cocceryl alcohol obtained from the three fractions mentioned above were purified by treatment with acetone in the following way. The material (37·7 g.) was stirred with 1200 ml. of acetone at 40° for a short time and filtered hot. The residue was recrystallised from boiling acetone and the product thus obtained again treated with 1200 ml. of acetone at 40°. After the fourth repetition of this operation the M.P. of the final residue (26 g.) was constant at 100·5–100·7°. Treatment of the acetone mother-liquor gave several fractions melting between 99° and 100° which have not yet been further purified.

Properties of cocceryl alcohol. The pure alcohol crystallises from most organic solvents, and particularly from acetone at 37°, in small colourless rhombic leaflets which melt sharply at 100·5–100·7° and set with a nucleus in long needles at 99·7°. (Found: C, 80·0, 80·0; H, 13·5, 13·4 %. $C_{34}H_{68}O_2$ requires: C, 80·2; H, 13·5 %.) It is insoluble in all organic solvents in the cold, is slightly soluble in boiling ether and low-boiling petroleum and is readily soluble in boiling benzene or boiling acetone.

Treatment with acetic anhydride and fused sodium acetate gave the *monoacetate*, M.P. 80·9–81·3°, which is soluble in ether and to a small extent in cold acetone. (Found: C, 78·3; H, 12·7 %. $C_{36}H_{70}O_3$ requires: C, 78·5; H, 12·8 %.) To find out if the presumed diacetate of Liebermann and Bergami [1887] was obtained through enolisation of the ketonic group we prepared the acetate according to their directions. 1 g. of the alcohol was heated with 0·5 g. of acetic anhydride in a sealed tube at 170° for 2 hours. The resulting product crystallised from ether-acetone in fine rhombic plates melting at 80·9–81·2° and was the monoacetate.

The *oxime*, prepared in the usual way and crystallised from alcohol, melted at 74·5–75°. (Found: C, 77·4; H, 13·5; N, 2·5 %. $C_{34}H_{68}O.NOH$ requires: C, 77·9; H, 13·3; N, 2·7 %.) It is readily soluble in cold ether and sparingly so in cold acetone.

Reduction of cocceryl alcohol to n*-tetratriacontanol.* 17·7 g. of cocceryl alcohol were reduced by Clemmensen's method [Le Sueur, 1915] using 160 g. of amalgamated zinc (2 portions) and 160 ml. of concentrated hydrochloric acid (changed six times). The reaction was allowed to proceed for 60 hours, when the acid was diluted with water and the reduced alcohol, together with the residual zinc, filtered off. The mixed product was treated with a large volume of boiling acetone, which was filtered hot. On cooling the filtrate gave 14·8 g. of reduced alcohol, M.P. 88·5–89·0°. A second treatment with hot acetone gave a further 3·2 g., M.P. 90·9–91·3°. The lower-melting fraction was recrystallised twice from acetone at 37° which raised the melting-point to 91·2–91·5°. The products were then combined and 10 g. acetylated with 12·8 g. of fused sodium acetate and 300 ml. of acetic anhydride. The acetate was washed with water, crystallised from acetone and then distilled. The bath temperature was 305°, and 6 fractions were collected at 220–235°/0·004 mm. On crystallisation from acetone they all melted at 75·5–75·8°. The collected material (7·7 g.) was saponified and the resulting alcohol crystallised twice from 4 litres of benzene-acetone (1:2) at 37° when the melting-point was constant at 91·9–92·2°. The yield was 5 g. (Found: C, 82·6; H, 14·2 %. $C_{34}H_{70}O$ requires C, 82·5; H, 14·3 %.) The large colourless rhombic plates obtained from acetone gave an excellent X-ray photograph (Series B, 266); up to 36 orders measuring 74·64 Å. in the B or short form, with no trace of the A or long form. The purity of the alcohol was checked by reduction *via* the iodide to *n*-tetratriacontane [Pollard *et al.*, 1931]. After treat-

ment with sulphuric acid this had M.P. 72·6–72·8°; S.P. 72·5°, and heating and cooling transition temperatures of 69·2–4° and 68·5° respectively, in excellent agreement with those of the pure synthetic paraffin [Piper *et al.*, 1931]. *n*-Tetratriacontanoic acid was prepared by oxidising the alcohol (0·5 g.) with chromium trioxide in glacial acetic acid in the usual way [Pollard *et al.*, 1931]. The crude acid was recrystallised twice from acetone at 37°, when it separated in large colourless rhombic plates, M.P. 98·3–98·5°. It gave an excellent X-ray photograph (Series B 270) in the B or long form measuring 80·4 Å., with no trace of the C or short form.

Oxidation of cocceryl alcohol to the corresponding ketonic acid. 13 g. of cocceryl alcohol dissolved in 3 litres of glacial acetic acid were treated with 7·2 g. of (moist) chromium trioxide at 65–70° for 20 minutes. The resulting green solution was poured with stirring into 7 litres of water and the solid which separated washed thoroughly with water. The salmon-pink powder thus obtained was dissolved in 2 litres of boiling 95 % alcohol, which was filtered hot. To the hot filtrate a further 200 ml. of boiling alcohol containing 10 g. of potassium hydroxide, followed by 300 ml. of boiling alcohol containing 15 g. of calcium chloride, were added. After boiling under reflux for 1 hour the mixture was filtered hot to separate the precipitated calcium soaps. These were extracted three times with 1500 ml. of boiling alcohol to remove any unchanged cocceryl alcohol and then dissolved in 600 ml. of boiling glacial acetic acid to decompose the soaps. The free ketonic acid was precipitated by pouring into excess of water, collected and washed repeatedly with water and cold acetone. It was then crystallised from 1500 ml. of acetone. The yield was 7·5 g. After recrystallisation from acetone at 37° the M.P. was 106·5–107°. (Found: C, 78·1; H, 12·9 %. $C_{34}H_{66}O_3$ requires: C, 78·1; H, 12·7 %.)

Oxime of the ketonic acid. 7 g. of the keto-acid, 7 g. of hydroxylamine hydrochloride and 12 g. of potassium hydroxide in 1500 ml. of 95 % alcohol were boiled under reflux overnight. The cooled solution was poured into 4500 ml. of dilute sulphuric acid. The solid which separated was extracted with 1200 ml. of cold ether, which was afterwards filtered. The ether was removed, and the solid residue crystallised from ice-cold acetone; 5·0 g. of oxime in microscopic needles, M.P. 62·5–63°, were obtained. (Found: C, 76·0; H, 12·6; N, 2·5 %. $C_{34}H_{66}O_2.NOH$ requires: C, 75·9; H, 12·7; N, 2·6 %.)

Transformation of the oxime and hydrolysis of the resulting amides. The procedure followed closely that observed with the oxime of 13-keto-*n*-dotriacontanoic acid. 4·5 g. of oxime gave 2·5 g. of mixed amides which were hydrolysed for 4 hours with concentrated hydrochloric acid in a sealed tube. The appropriate treatment gave ether-alcohol-insoluble material weighing 0·67 g. (Fraction A); a very small amount of benzene-alcohol-insoluble material (Fraction B) which was neglected, alcohol-insoluble soaps (Fraction C) and alcohol-soluble soaps (Fraction D).

Fraction A. This was extracted twice with boiling benzene. On cooling, the benzene deposited 0·28 g. of crystalline material. This was recrystallised from benzene, extracted thoroughly with ether and the residue crystallised from a mixture of 20 ml. of ether and 10 ml. of alcohol. 0·11 g. of *n*-nonadecanamine hydrochloride were obtained. (Found: C, 71·4; H, 13·3; N, 4·2 %. $C_{19}H_{41}N.HCl$ requires: C, 71·3; H, 13·1; N, 4·4 %.) The benzoate, alone and mixed with the synthetic product, melted at 88°.

The material originally insoluble in benzene melted at 157–159°. After recrystallisation first from water and then from 2*N* hydrochloric acid it melted at 166°. The crystalline form and solubilities were similar to those of the

12-aminolauric acid hydrochloride mentioned above, and there was no doubt that it was the expected, but unknown, 14-aminomyristic acid hydrochloride. Again we found the amino-acid very difficult to purify, and after repeated treatment with boiling acetone and boiling benzene there remained only 18 mg. of material melting at 168°. (Found: (micro-Kjeldahl) N, 4·7 %. $C_{14}H_{31}N.HCl$ requires: N, 5·0 %.) 13 mg. were dissolved in hot water and the requisite amount of alkali added. The free acid crystallised on cooling in thin leaflets, M.P. 181–182°. There was insufficient material for further treatment or analysis. In a mixed melt with 12-aminolauric acid (M.P. 183°) the M.P. was depressed to 168–169°.

Fraction C. The soaps were dissolved in acetic acid, which was then diluted with water. The free acids were collected and crystallised from aqueous acetone. The material thus obtained was extracted with light petroleum (B.P. < 40°). The extract on concentration yielded 38 mg. of fatty acid, M.P. 68°. This and the insoluble residue were joined with the similar products isolated from Fraction D.

Fraction D. This was treated in a similar way to Fraction C. The material soluble in cold petroleum (60 mg.), with the added material mentioned above, was recrystallised from alcohol, acetone and finally from light petroleum. It proved to be *n*-eicosanoic acid. (Found: C, 77·2; H, 12·8 %; mol. wt. by titration 308. $C_{20}H_{40}O_2$ requires: C, 76·9; H, 12·9 %; mol. wt. 312.) It melted at 74·0–74·2°, and in a mixed melt with the synthetic acid (M.P. 75–75·2°) the M.P. was 74·6–74·8°.

The residue insoluble in cold petroleum was extracted with boiling water, which was filtered hot. On cooling, a few mg. of a white crystalline solid separated, M.P. 111–112°. It was thought that this acid would be *n*-tridecane-(1:13)-dicarboxylic acid, M.P. 115°, which has been recently synthesised by Chuit [1926]. As purification of the brassylic acid in the preceding case had proved exceptionally troublesome, and as none of the synthetic acid was available for a mixed melt, the small amount of material was boiled under reflux in 5 ml. of methyl alcoholic hydrogen chloride to prepare the dimethyl ester. This melted at 42°, alone and mixed with the synthetic ester (M.P. 43°) kindly supplied by Dr Chuit. There was insufficient material for analysis.

*15-Hydroxy-*n*-tetratriacontanol.* Cocceryl alcohol (1 g.) in boiling amyl alcohol (100 ml.) was reduced with sodium (8 g. in small portions). The product was washed with water and recrystallised twice from acetone (350 ml.) at 37°, when it was obtained in small colourless rhombic plates, M.P. 98·9–99·1°. The yield was 0·7 g. (Found: C, 80·1; H, 13·8 %. $C_{34}H_{70}O_2$ requires: C, 79·9; H, 13·8 %.) The diacetate was prepared with fused sodium acetate and acetic anhydride in the usual way and recrystallised twice from a small volume of acetone at 0°, when it separated in small rosettes of colourless needles. The melting-point was not sharp, the material became molten at 49° and the melt cleared at 50–51°. (Found: C, 76·6; H, 12·4 %. $C_{38}H_{74}O_4$ requires: C, 76·8; H, 12·5 %.) It is fairly readily soluble at room temperature in ether and acetone.

Summary.

Coccerin, the benzene-soluble, alcohol-insoluble wax of the cochineal insect (*Coccus cacti*) has been investigated in some detail.

The cocceryl alcohol of Liebermann is not a dihydric alcohol containing 30 carbon atoms as he suggested but is 15-keto-*n*-tetratriacontanol. His coccerinic acid is a mixture consisting of 13-keto-*n*-dotriacontanoic acid and *n*-triacontanoic acid.

The generic relationship between the ketonic acid and ketonic primary alcohol present in this wax with the secondary alcohol *n*-nonacosan-10-ol present in the wax of apple cuticle is briefly discussed.

Reasons are given for the suggestion that all previous work on the constitution of insect waxes is in need of reinvestigation.

In conclusion we should like to thank Dr S. H. Piper for several X-ray analyses which have materially aided us in the course of this work; Mrs G. M. Robinson for a sample of 11-bromoundecanoic acid; Chuit, Naef and Co., Geneva, for a sample of methyl *n*-tridecane-(1:13)-dicarboxylate; Dr N. K. Adam for samples of 12-aminolauric acid and brassylic acid prepared by the late Dr Le Sueur; Mr A. L. Bacharach of Glaxo, Ltd., for kindly arranging for the extraction of 15 kg. of cochineal with boiling benzene and Dr F. H. Carr of British Drug Houses for a similar extraction of 60 kg. of cochineal.

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XLIV. THE PROTEINS OF GRASSES.

III. THE CYSTINE CONTENT OF CERTAIN GRASSES AND OTHER PASTURE PLANT PROTEINS.

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(*Received January 8th, 1934.*)

A few years ago Robertson [1928] suggested that since the keratin of wool contained such a high proportion of cystine (about 13 %) the carrying capacity of any country for sheep which are grown for wool production might well be determined by the capacity of its pasture plants to produce cystine. Attention thus became focused on the cystine content of grasses and other pasture plants. Now the major part of this cystine will presumably be present as a constituent of the leaf proteins, and to determine this protein-cystine it is necessary to hydrolyse the protein with strong acid or (less satisfactorily) to digest it with appropriate proteolytic enzymes. As no method for preparing these proteins has hitherto been available earlier workers have found it necessary to submit the whole grass or pasturage itself to acid hydrolysis or to enzymic digestion. This means, in effect, that the proteins in the leaf material have been treated in the presence of from three to six times their own weight of cellulose, sugars, organic acids *etc.*, and it is not at all surprising that under these very unsatisfactory conditions the highly specific colour reaction of Sullivan for cystine was found difficult to carry out and indicated the presence of only a trace of this amino-acid [Aitken, 1930; Evans, 1931; Henrici, 1932]. Evans [1931] also, assuming the alkali-labile sulphur to represent two-thirds of the cystine-sulphur, found that the various samples of pasturage examined by him might contain from 0·07 to 0·18 % of cystine on the basis of dry matter. He pointed out however that not all the labile sulphur of proteins can correctly be ascribed to cystine and suggested that the true cystine content of grass must be less than 0·1 % of the dry matter.

Rimington and Bekker [1932], adopting the figure 0·01 % of dry matter as a fair average value for the cystine content of grass, calculate that a sheep producing a 12 lb. fleece, which contains about 0·78 lb. of cystine, would have to eat 21·4 lbs. of dry grass daily in order to obtain this amount of preformed cystine. Since sheep under optimum conditions will consume only 4 lbs. of dry matter daily they conclude that grasses are deficient in cystine. Woodman and Evans [1932] disagree with this conclusion, and point out that Evans's value, 0·1 % of the dry matter, for the cystine content of cultivated English pasturage would enable sheep to secure the cystine necessary for a 12 lb. fleece during a grazing period of only 195 days. Rimington *et al.* [1933] maintain, however, that the value of 0·1 % for the cystine is too high, and in support of their contention instance a stud ram which produced a fleece of 46 lbs. on a diet consisting almost entirely of lucerne.

These discussions were based on inadequate experimental evidence. During the past three years methods have been perfected in this laboratory for pre-

paring the proteins of grasses and other forage plants, and we have now completed a fairly searching enquiry into their cystine content. We first determined cystine by the well-known methods of Sullivan. The protein hydrolysates were, however, deeply coloured and could not be satisfactorily decolorised by means of norite or kaolin without large losses of cystine. Our results with cocksfoot protein, which we used exclusively in our preliminary experiments, suggested only 0·1–0·2 % of cystine.

The method of Vickery and White [1933], however, details of which were kindly supplied to us some months before publication by Dr Vickery, gave very much greater values, 1·1–1·2 %. According to this method the protein is hydrolysed in the presence of tin, which reduces the cystine to cysteine as it is set free. The cysteine is then precipitated from solution under appropriate conditions as the very insoluble cuprous mercaptide. This precipitate is decomposed with hydrogen sulphide, the cuprous sulphide removed and the organic sulphur in the clarified solution determined. In all the cases examined by them the cystine content of the protein calculated from this sulphur value agreed reasonably well with the content determined by Sullivan's procedure. In the case of our leaf proteins, as mentioned above, there was a great discrepancy. Moreover the value for cystine determined by Sullivan's method on the clear filtrate from the cuprous sulphide was similar to that obtained on direct hydrolysis. Clearly the Vickery and White procedure was estimating, in addition to the sulphur of cystine, the sulphur of (1) an unknown amino-acid giving an insoluble cuprous salt, or (2) products arising from the decomposition of cystine during the acid hydrolysis of the protein in the presence of the small amount of impurity which all our grass protein preparations contain.

We have been able to eliminate the latter alternative by making use of the new modification of Sullivan's method introduced by Prunty [1933]. The cystine in the hydrolysate is reduced to cysteine by means of zinc in acid solution instead of with sodium cyanide, and during the reduction the hydrolysate is almost completely decolorised. Accurate and easily reproducible values for the cystine content of the leaf proteins were readily obtained. Added cystine could also be recovered in 90–95 % yield, showing that no appreciable destruction of cystine was taking place during hydrolysis. Additional proof was afforded by experiments in which the protein was hydrolysed by enzymes.

One very interesting result of our research has therefore been the clear proof that leaf proteins must contain at least one unknown sulphur-containing amino-acid, because Vickery and White have shown that methionine, which is present in these proteins, is not precipitated under their conditions as a cuprous salt. This unknown amino-acid yields part of its sulphur as sulphide on alkaline hydrolysis and may quite likely be the same product which the experiments of Zahnd and Clarke [1933] show to be present in egg-white.

The cystine content of the pasture leaf proteins, determined by the Prunty-Sullivan colorimetric method, varies between 0·25 and 1·25 %, as shown in Table I, and by calculation it is possible to show quite definitely that a mixed herbage of the type which constitutes the pasturage of sheep in this country contains an adequate amount of cystine for the production of a 12 lb. fleece. Lucerne protein has also been examined, and as it is relatively rich in cystine there is no reason to doubt that the prize ram referred to by Rimington *et al.* [1933] also obtained all the necessary cystine from its diet. As the sulphur of the unknown amino-acid referred to above is not stable to alkali it is clear that Evans's determinations of labile sulphur in English pasture grasses were not a valid basis for calculating their cystine contents.

Experimental.

Preparation of the proteins. Full details have been given in a previous paper [Chibnall *et al.*, 1933]. The nitrogen content of the various preparations ranged from 10 to 15 %, owing to the presence of non-nitrogenous impurities whose chemical composition is not yet clearly understood [Miller and Chibnall, 1932]. The percentage of cystine determined on these preparations therefore falls short of the percentage in the protein by an amount depending on the proportion of these impurities present. Leaf proteins known to be pure contain from 15·7 to 16·7 % N [Chibnall and Grover, 1926]. On the assumption that the grass and other pasture plant proteins should also contain 16 % N we have, in the Tables given below, calculated the percentage of cystine in the protein on this basis. Adoption of this figure has the additional advantage that in our subsequent discussion on the cystine content of pasture in relation to wool production we are able to make direct comparison with the conventional protein (N × 6·25) of the agricultural chemist.

Colorimetric estimation of cystine by the Prunty modification of Sullivan's method. The protein (0·5–1·0 g.) was heated with 25 ml. of 20 % hydrochloric acid in a flask immersed in a boiling water-bath for 2 hours, and then gently boiled under reflux overnight. The flask was cooled in ice water, 40 % aqueous sodium hydroxide added to p_H 2·5 and the precipitated humin separated from the yellow or orange solution by centrifuging. To the humin remaining in the centrifuge-tube 5–10 ml. of dilute sodium chloride solution previously adjusted to p_H 2·5 were added, and the tube was immersed in a boiling water-bath for 10 minutes, the mixture being frequently stirred. The humin was again centrifuged off and the washing repeated at least eight times in all. Further washings gave no appreciable amount of cystine by the colorimetric method. The use of aqueous sodium chloride instead of water for washing the humin was necessary to prevent the formation of colloidal suspensions. The final solution was brought to a convenient volume, usually 100 ml., the last few washings being concentrated, if necessary, *in vacuo*.

Two portions of 5 ml. were measured into centrifuge-tubes, and to each was added 1 drop of concentrated hydrochloric acid and a few mg. of zinc dust. The tubes were then heated in a boiling water-bath for 5 minutes, cooled and centrifuged. The faintly yellow solutions were decanted into 15 ml. standard flasks, containing 1 ml. of 25 % aqueous sodium hydroxide and 2 ml. of 10 % aqueous sodium cyanide, the tubes being washed out with the requisite amount of water to make up the standard volume. At the same time a convenient amount of cystine was reduced and treated in a similar way. To one of the unknown solutions (A) and to the known cystine solution (S) were added 1 ml. of a 1 % solution of sodium β-naphthoquinonesulphonate and then immediately 5 ml. of a 10 % solution of sodium sulphite in 0·5 *N* aqueous sodium hydroxide; while to the other unknown (B) was added only the sulphite solution. After remaining at 25° for 30 minutes to each of the three solutions was added 1 ml. of a 2 % solution of sodium hydrosulphite in 0·5 *N* aqueous sodium hydroxide. The colour of a known depth of solution (A) was then compared with the colour of the same depth of the blank solution (B), superimposed on a variable depth of the standard solution (S), using a Klett colorimeter. No rapid fading of the red colour has been observed with our hydrolysates such as that recorded by Prunty in the case of gelatin. After standing in the air for half an hour the solutions become slightly darker, but the addition of a very small amount of sodium hydrosulphite at once restores the original colour. The results for the pasture leaf proteins are fully set out in Table I.

Table I. *The cystine content of pasture leaf proteins estimated by the Prunty modification of Sullivan's method.*

Species	Sample of leaf protein: Total N (ash-free) %	Sample of leaf protein: Cystine %	True protein (N = 16 %) Cystine %
Red clover (*Trifolium pratense*)	12·6	0·16	0·21
Bent (*Agrostis* sp.)	10·3	0·15	0·24
Cocksfoot (*Dactylis glomerata*) (AB)	14·6	0·26	0·28
,, (AC)	13·4	0·20	0·24
,, (AD)	13·2	0·20	0·24
,, (AG)	13·2	0·21	0·26
,, (AH)	13·5	0·23	0·27
Rough-stalked meadow grass (*Poa trivialis*)	13·8	0·23	0·27
Wild white clover (*Trifolium repens*)	13·2	0·26	0·32
Timothy (*Phleum pratense*)	13·8	0·29	0·34
Perennial ryegrass (*Lolium perenne*)	12·7	0·38	0·48
Yorkshire fog (*Holcus lanatus*)	11·8	0·36	0·49
Italian rye-grass (*Lolium italicum*)	14·0	0·59	0·67
Tall fescue (*Festuca elatior*)	13·6	0·60	0·70
Lucerne (*Medicago sativa*) (A)	14·4	0·66	0·73
Crested dog's tail (*Cynosurus cristatus*)	14·9	0·70	0·75
Meadow fescue (*Festuca pratensis*)	13·9	0·67	0·80
Red fescue (*Festuca rubra*)	14·2	0·77	0·87
Hard fescue (*Festuca duriuscula*)	14·6	0·85	0·90
Chewings fescue (*Festuca rubra* var. *Fallax* (Hack.))	14·0	0·85	0·93
Lucerne (*Medicago sativa*) (B)	15·8	1·20	1·20

Possible losses in the colorimetric estimation of cystine. The possibility that the impurities present in our protein preparations were causing extensive breakdown of cystine was investigated by adding a known amount of this amino-acid before hydrolysis. The results of two typical experiments with cocksfoot and crested dog's tail proteins are given in Table II, and show a recovery of added

Table II. *Showing the recovery of added cystine.*

Protein preparation	Cystine %	Cystine added %	Total cystine estimated %	Recovery of added cystine %
Standard HCl hydrolysis (Prunty):				
Cocksfoot (AC)	0·19	0·15	0·31	91
,,	0·19	0·50	0·65	94
Crested dog's tail	0·70	0·70	1·36	97
,,	0·70	0·70	1·34	95
Hydrolysed with H_2SO_4 in presence of tin:				
Cocksfoot (AB)	0·25	—	0·21	84
,,	0·25	0·63	0·74	84

cystine in each case of over 90 %. Lugg [1933] has shown that cysteine, produced from cystine by heating with stannous chloride and hydrochloric acid, may suffer almost complete decomposition if hydrolysed in the presence of relatively large amounts of sugars and amino-acids. With pure proteins Vickery and White [1933] experienced no such loss. As hydrolysis with hydrochloric acid in the absence of oxygen [Okuda, 1926] showed that cocksfoot protein contained about 0·1 % of cysteine we think it worth while to record the following experiment.

Two portions of 1 g. of cocksfoot protein (AB) were taken, and to one were added 6·3 mg. of cystine. Each portion was then hydrolysed with 20 ml. of 8*N* H_2SO_4 for 20 hours in the presence of 1 g. of granulated tin. The solutions were

cooled, 40 % aqueous sodium hydroxide was added to p_H 2·5 and the mixtures, each containing the humin in suspension, were saturated with hydrogen sulphide. The resulting pale yellow solutions were cleared by centrifuging, and the residue of stannous sulphide and humin was washed eight times successively with dilute hydrochloric acid. In neither case did the last two washings give any nitroprusside reaction. The solutions were concentrated *in vacuo* and the cystine+cysteine present estimated colorimetrically in the usual way. The results are given in Table II. If it be assumed that the cystine content of the protein be that given on standard hydrochloric acid hydrolysis, then the amount of cystine estimated in each case amounted to 84 %. The loss is possibly due in part to the fact that air oxidation of cysteine to cystine (which must have taken place to a small extent during the concentration *in vacuo*) is not strictly quantitative, but we think that the major loss is due to adsorption of cysteine on the stannous sulphide; even greater losses (20–30 %) were found by Lugg [1933] when cystine was heated with stannous chloride and hydrochloric acid, the metal being subsequently removed as sulphide. The results show fairly conclusively that Prunty's modification of the Sullivan method estimates 90–95 % of the total cystine and cysteine present in the pasture leaf proteins.

The alkali-labile sulphur as a measure of cystine content. The procedure employed by Osborne [1902] was followed. The results were somewhat erratic, as Osborne found with seed proteins, but representative values for the alkali-labile sulphur of cocksfoot protein are given in Table III. If we accept Osborne's conclusion that this represents about two-thirds of the total cystine-sulphur, the value for cystine in each case would be approximately three times that given by the Prunty-Sullivan method. It appears from this result that a determination of alkali-labile sulphur estimates not only the sulphur of cystine but also in large part the sulphur of the unknown sulphur-containing amino-acid whose presence in the hydrolysis products of these leaf proteins is clearly demonstrated by the Vickery and White procedure outlined below. It follows therefore that the cystine values for pasture grasses quoted by Woodman and Evans [1932], which are based on determinations of alkali-labile sulphur by Evans [1931], are not valid.

Table III. *Showing the distribution of organic sulphur in certain pasture leaf proteins.*

		True protein (N = 16 %)				
Species	Protein preparation Total N (ash-free) %	Cystine-sulphur (Sp) (Prunty-Sullivan) %	Cystine-sulphur (Sv) (Vickery and White) %	Sv − Sp	Alkali-labile sulphur %	Total sulphur %
Cocksfoot (AB)	14·6	0·072	0·26	0·19	0·12	1·10
,, (AC)	13·4	0·061	0·31	0·25	0·15	1·30
,, (AG)	13·2	0·063	0·31	0·25	—	1·40
Tall fescue	13·6	0·184	0·29	0·11	—	1·21
Red fescue	14·2	0·221	0·42	0·20	—	1·56
Hard fescue	14·6	0·24	0·38	0·14	—	1·85
Perennial ryegrass	12·7	0·125	0·26	0·14	—	1·56
Lucerne	14·4	0·19	0·28	0·09	—	1·55

Estimation of cystine(mercaptide)-sulphur by the method of Vickery and White [1933]. 5–10 g. of protein were hydrolysed in the presence of tin and the cysteine precipitated as the cuprous mercaptide. The procedure of these authors gave

consistent results without difficulty, and, as will be seen from Table III, the values for cystine(mercaptide)-sulphur are in all cases greatly in excess of those obtained by the Prunty-Sullivan colorimetric method. If the latter represent the true cystine-sulphur, as we believe, then it follows that the cuprous oxide must have precipitated at least one other sulphur-containing amino-acid. Confirmation of the low cystine content of the cuprous precipitate was obtained in the following experiment.

46 g. of hard fescue protein, containing 0·84 % of cystine by the Prunty-Sullivan colorimetric method, were treated according to the procedure of Vickery and White. The cuprous mercaptide was then decomposed with hydrogen sulphide in the presence of sulphuric (not hydrochloric) acid. The cuprous sulphide was centrifuged off and washed repeatedly by boiling with dilute sulphuric acid until the washings gave no nitroprusside reaction for cysteine. The clean solution and washings were united, adjusted to p_H 7 with barium hydroxide and aerated for 1 hour, after which time no free cysteine could be detected by the nitroprusside reaction. The solution was then made slightly acid with sulphuric acid and concentrated *in vacuo*. The precipitate of barium sulphate was centrifuged off and washed repeatedly with 0·002 *N* sulphuric acid. The solution and washings were united and concentrated *in vacuo* to 250 ml. Cystine, by the Prunty-Sullivan method, amounted to 0·27 g., equivalent to 0·6 % of the protein taken for analysis. Vickery and White show that a certain amount of cysteine destruction occurs during the decomposition of the cuprous mercaptide. This amount of cystine contains 0·072 g. of sulphur, whereas the total organic sulphur present was 0·173 g.

The solution was then concentrated *in vacuo* to 25 ml. and an equal volume of alcohol added. The pale brown precipitate which came out in the course of a few days was filtered off, dissolved in a few ml. of dilute hydrochloric acid and a small residue of barium sulphate filtered off. The clear filtrate was then cooled to 0° and saturated with hydrogen chloride gas. The mass of fine needle-shaped crystals, typical of cystine dihydrochloride, which separated overnight, was filtered off and washed with a little cold absolute alcohol. The weight was 0·14 g., and the mother-liquor on treatment gave a further 0·06 g., making 0·22 g. in all, a yield of 60 % of that estimated colorimetrically. For analysis (Schoeller) the material was dried at 60° *in vacuo* over phosphorus pentoxide. (Found: S, 20·6, 20·4 %. $C_6H_{14}O_4N_2S_2Cl_2$ requires: S, 20·45 %.) The yield of cystine compares favourably with that of Vickery and White, if it be remembered that our solutions contained nearly an equal amount of another sulphur-containing amino-acid.

The liberation of cystine during enzymic digestion. Additional evidence that the Prunty procedure gives a true estimate of the cystine content of the proteins, and that the Vickery and White procedure is not estimating any decomposition products of cystine, was obtained from experiments in which the protein was broken down by enzymic digestion and was therefore not subject to the action of products set free from the non-protein impurities by the strong acid hydrolysis. Six digests of cocksfoot protein and one of Italian ryegrass protein have been examined; we shall however only quote one result of the former. Details of the experiments follow closely those concerned with the amide-N of proteins [Damodaran, 1932, Damodaran *et al.*, 1932], the enzymes used being pepsin, trypsin and intestinal (pig) erepsin in succession.

The following account concerns the digestion of cocksfoot protein (AB). (Data corrected for added enzyme preparation.) The protein (9·5 g.) was suspended in 400 ml. of dilute hydrochloric acid (p_H 1·9) containing 0·4 g. of pepsin.

The protein rapidly settled, and the flask was gently shaken at intervals during 41 hours. At the end of that time much of the protein still remained undissolved, the supernatant liquid containing only 16·6 % of the total N. Previous digests had shown that no appreciable amount of cystine was liberated by pepsin [*cf.* Jones and Gersdorff, 1933]. *N* sodium hydroxide solution was then added to p_H 8·2, when the major part of the protein residue was at once dissolved. 0·4 g. of trypsin was next added, together with 0·5 % of tricresol as antiseptic. It was necessary to adjust the digest to p_H 8·2 every few hours. Cystine and amino-N were determined at intervals, as shown in Table IV. At the end of 72 hours' digestion, when no appreciable protein residue remained, 40 ml. of pig erepsin were added. The solution was maintained at p_H 8·0, and the amino-N reached a maximum at the end of a further 17 hours. In the case of cocksfoot no appreciable increase of cystine was observed with erepsin, though a definite increase was noted in the case of Italian ryegrass. All the enzymic digests were very deeply coloured, and on neutralisation to about p_H 2·5 a large amount of material, partly humin and partly of a proteose nature, was thrown down and was difficult to wash. The cystine determinations were therefore not as consistent or as reliable as those obtained with corresponding acid hydrolysates. It was observed in each case that if the digestion with either trypsin or erepsin were prolonged the cystine value fell slowly from the maximum, indicating a slow secondary decomposition of cystine under the conditions of the experiments.

The results however leave no doubt that the amount of free cystine liberated was of the magnitude that one would expect if the total cystine in the protein were that given by the Prunty-Sullivan colorimetric method and not that given by the Vickery and White mercaptide method; in the case of the cocksfoot protein quoted in Table IV the liberation of cystine was 70 % and of amino-N was 58 % of the maximum given on acid hydrolysis, while with the Italian ryegrass the corresponding figures were 62 % and 64 % respectively.

Table IV. *Showing the amount of cystine set free during the enzymic digestion of cocksfoot and Italian ryegrass proteins.*

		Cocksfoot protein (AB) 14·6 % N 0·25 % cystine		Italian ryegrass protein 14·0 % N 0·58 % cystine	
Enzyme	Duration of digestion (hours)	Cystine (% total protein)	Amino-N (% total N)	Cystine (% total protein)	Amino-N (% total protein N)
Pepsin	41	—	2·4	—	—
Trypsin	24	0·09	—	0·20	—
,,	48	0·10	—	0·24	—
,,	72	0·18	28	0·30	34·8
Erepsin	17	0·17	40·2	0·36	44·8
,,	41	0·15	40·2	0·33	45·0

The total organic sulphur of the proteins. The experimental data given above show that the hydrolysis products of all the pasture leaf proteins examined contain cystine, and that this can be readily estimated by the highly specific colorimetric method of Sullivan. They show also that another amino-acid, probably one containing a sulphydryl group, must also be present. It will be seen from Table III, however, that these two amino-acids, both of which are estimated by the method of Vickery and White, account for only about one-quarter of the organic sulphur present in the proteins. An estimation of methionine on cocksfoot protein by the indirect method of Baernstein [1932]

suggests that the remainder may be due to this amino-acid. Further investigations into the constitution of the unknown amino-acid and of the methionine content of the proteins are in progress.

Reference to Table II shows that in the case of cocksfoot protein with a low cystine content the Prunty-Sullivan method estimates only 90–95 % of the total amount present. If the protein preparation were very impure, such as that of bent or yarrow (N = 10 %), it is probable that the loss could be even greater. When preparing the data for Table V, therefore, we have in these cases made what we consider a legitimate allowance for a possible loss during the estimation of from 5–15 % of the total cystine. We have in a few instances determined the cystine content of the dried plant material itself by the Prunty-Sullivan procedure described above. After the reduction process the solutions are somewhat darker than in the case of the proteins, but the red colour subsequently developed by the cysteine can be readily estimated. Low values were always obtained, and added cystine was destroyed to the extent of 40–50 %. This result was not unexpected.

The cystine content of pastures and wool production. Table V gives the calculated amount of cystine, in terms of total dry matter, present in the various

Table V. *Cystine content of pasture plants at various protein levels.*

Species	Percentage of cystine in protein (N = 16 %)	Cystine as a percentage of total dry matter in the herbage, containing					
		Protein 8 %	Protein 12 %	Protein 16 %	Protein 20 %	Protein 24 %	Protein 28 %
Yarrow, red clover	0·25	0·02	0·03	0·04	0·05	0·06	0·07
Bent, cocksfoot, rough stalked meadow grass	0·30	0·024	0·036	0·048	0·06	0·072	0·084
Timothy, wild white clover	0·35	0·028	0·042	0·056	0·07	0·084	0·098
Perennial ryegrass, Yorkshire fog	0·50	0·04	0·06	0·08	0·10	0·12	0·14
Italian ryegrass, tall fescue	0·70	0·056	0·084	0·112	0·14	0·168	0·196
Lucerne (A)	0·75	0·06	0·09	0·12	0·15	0·18	0·21
Crested dog's tail, meadow fescue	0·80	0·064	0·096	0·128	0·16	0·192	0·224
Red fescue, hard fescue	0·90	0·072	0·108	0·144	0·18	0·216	0·252
Chewings fescue	0·95	0·076	0·114	0·152	0·19	0·228	0·266
Lucerne (B)	1·2	0·096	0·144	0·192	0·24	0·288	0·336

pasture plants at certain definite protein levels. These values are based on the assumption that the protein is the sole source of cystine. They must therefore be regarded only as minimum values, because cystine or cystine peptides may possibly be present in the water-soluble, non-protein products of the plants, and these have not yet been investigated.

Before attempting to assess the value of these plants as diet for sheep it is necessary to make one or two observations concerning the actual samples which were used for preparing the proteins. All the grasses were taken from plots which had been cut at least once a fortnight, and the samples consisted almost entirely of blades. The red clover and lucerne (A) were each cut about 1 inch above ground, and both samples consisted of somewhat stunted plants with relatively few leaflets. The protein preparations therefore consist of leaf protein well adulterated with that from the stems. The wild white clover and lucerne (B),

on the contrary, were from luxuriant crops, and both were cut with a scythe held well above the ground so that the sample consisted chiefly of leaflets obtained from the upper few inches of the plants. The protein preparations, therefore, are very rich in the leaf protein. This accounts for the striking difference between the two samples of lucerne protein. Sample A, which contained 14·4 % N and 0·73 % cystine, was prepared from lucerne containing 17·2 % true protein. Sample B, which contained 15·8 % N and 1·2 % cystine, was prepared from lucerne grown in Connecticut, U.S.A., containing about 28 % true protein [Chibnall and Nolan, 1924].

With regard to the cystine content of pastures and wool production the data given in Table V can only be discussed in general terms because in actual practice the herbage is always mixed and may change considerably in botanical composition during the season. Furthermore, the question of the amount of ingested cystine which is catabolised or excreted is outside the scope of this paper, so that in the following discussion we can do no more than attempt to show that the minimum amount of cystine ingested is greater than that required for wool-protein synthesis.

If we assume that a sheep eats pasturage containing 4 lbs. of dry matter daily, then the 0·39 lb. of cystine necessary for an average British fleece of 6 lbs. would be secured during one year on a diet containing only 0·0265 % of cystine on the basis of dry matter. This is well below the amount which would be provided by the poorest of pasture consisting chiefly of bent, even if we ignore the fact that such pastures would generally contain a small proportion of one of the fescues, which are rich in cystine. The winter grazing (8 % protein) might be on the borderline, but for 6–8 months during the growth period the protein level would exceed 12 %, and the cystine content of the diet would be in excess of 0·04 %. A 12 lb. fleece, which is the weight discussed by Rimington and Bekker [1932], contains 0·78 lb. of cystine, and for this intake the sheep would require a diet throughout the year containing 0·053 % of cystine on the basis of dry matter. In Great Britain a cultivated pasture of the type which might be expected to carry sheep bearing such a fleece would probably consist in large part of perennial ryegrass, with subordinate amounts of Italian ryegrass, rough-stalked meadow grass, wild white clover and bent at the appropriate season. From March to October the pasture would contain on an average nearly 20 % of protein on a basis of dry matter, so that the cystine content of the diet throughout this period would vary between about 0·08 and 0·1 %. This grazing period alone therefore would provide more than enough cystine for the fleece. During the winter months the protein content of the grazing would fall, but the early appearance of Italian ryegrass would help materially in maintaining an average cystine content in the diet of about 0·05–0·06 %. From an agricultural point of view these descriptions of poor and good quality pastures are necessarily very incomplete, and they are used here only as simplified examples to illustrate our general contention that the pastures of Great Britain almost certainly contain considerably more cystine than the sheep, which they normally carry, require for wool-protein synthesis, especially if it be remembered that sheep will always eat preferentially the young tips of the leaves, which are the richest in protein and therefore in cystine.

In many countries lucerne meal forms one of the principal winter rations of sheep. This has a protein content of about 15 % and is made from cuttings of the whole plant, corresponding to lucerne (A) in Table V. It would therefore have a cystine content of about 0·12 %. If it contained proportionately more leaf material than our sample, which is very probable, then the cystine content

at this protein level would approach the value 0·19 % for lucerne (B) in Table V. These figures are very high indeed when compared with the minimum intake required for the 6 and 12 lb. fleeces mentioned above.

The South African stud ram referred to by Rimington *et al.* [1933], which clipped a record fleece of 46 lbs., was stated to have been fed almost entirely on lucerne. Such a fleece would contain about 3 lbs. of cystine, and on a diet containing 4 lbs. of dry matter daily this amount of cystine would be consumed in one year if the lucerne contained 0·21 % of cystine. This is higher than the value one would expect from ordinary lucerne meal, but a prize stud animal of this type would undoubtedly be given the best possible diet. During the appropriate season it would be turned out to feed on fresh lucerne, and it would undoubtedly choose in preference the leafy lucerne tops. One of us (A. C. C.) analysed many samples of such material collected during the summer of 1924 from crops growing around New Haven, Connecticut, U.S.A., and the true protein content in all cases was never less than 24 % and generally 30 % of the total dry weight. Such material would contain from 0·25–0·3 % of cystine. Lucerne leaf-meal, containing 20–24 % of protein, and hence 0·24–0·27 % cystine, might have formed part of the diet in the stall. In the absence of precise data on the ram's diet, therefore, a cystine intake more than sufficient even for a 46 lb. fleece does not appear extravagant.

Summary.

The cystine content of several typical grass and other pasture plant proteins has been determined by Prunty's modification of Sullivan's colorimetric method. The grass proteins all contain cystine, in amount varying from 0·3 to 0·95 %. Lucerne leaf protein is particularly rich in cystine and contains 1·2 %.

The gravimetric method of Vickery and White gives in all cases a much higher value for cystine-sulphur than the amount shown to be present and must include the sulphur of an unknown amino-acid.

The protein-cystine content of the grasses and other pasture plants at various protein levels has been calculated, and it appears that pasturage contains ample cystine for the wool-protein requirements of the sheep which it normally carries.

We should like to record our thanks to the Imperial Chemical Industries, Ltd., for a grant to cover the cost of this research, to their staff at Jealott's Hill for many helpful discussions and to Mr R. G. Westall for analytical assistance.

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XLV. THE PENETRATION OF ERYTHROCYTES BY ANIONS.

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(*Received January 8th, 1934.*)

THE unequal penetration of ions into erythrocytes has been the subject of numerous observations. Thus the mature cell although impermeable to cations, contains large quantities of potassium, and this in spite of its being suspended in plasma which contains little potassium and much sodium. Again, though erythrocytes are permeable to anions the latter show varying degrees of penetration, and even anions which permeate freely, frequently show an anomalous distribution; the concentration of normal cell-bicarbonate is higher than would have been anticipated from a simple Donnan equilibrium, while Hastings and Van Dyke [1931] have shown that a similar excess of bromide occurs, if bromide is added to the fluid in which erythrocytes have been suspended.

The present investigation is concerned with the entry into erythrocytes of various acids or anions and attempts to correlate certain physical properties with their ability to penetrate the cell membrane.

The relative permeation of anions may be determined from the amount of one anion entering the cell in a given period of time compared with the amount of a second anion entering during the same period. Alternatively, one can expose erythrocytes to a solution containing two salts and estimate their relative concentrations in the cells. The second method has been adopted chiefly because differences in anion permeation, which are barely perceptible with cells suspended in solutions of single salts, are greatly magnified in solutions of the mixed salts, the more penetrating ion greatly depressing the entry of the less penetrating ion. Chloride was adopted as the standard anion and the second anion B was estimated indirectly and sometimes both directly and indirectly. The indirect method is based on the following assumptions:

(1) *If erythrocytes are suspended in KCl solutions, chloride diffuses across the cell membrane and equilibrium is reached within five minutes at 23°.* The validity of this assumption is shown by the figures in Table I.

Table I.

	External p_H 5·5 Ext. Cl 205 m.eq.			Ext. p_H 9 Ext. Cl 88 m.eq.	
Time	% of original volume (V)	Cl (m.eq.)	Time	(V)	Cl (m.eq.)
0 min.	100	50	0	100	50
1 „	94	141	1	140	22
5 „	102	165	5	122	20
60 „	102	170	60	122	20

(2) *The amount of chloride entering erythrocytes from pure KCl solutions depends only on* p_H. This statement is approximate. The erythrocyte contains

haemoglobin and potassium which are unable to penetrate the cell membrane, and according to the Donnan equation, if the external Cl falls, some decrease of cell-Cl must also occur. The actual decrease of Cl however is barely detectable by ordinary analytical methods, and if at the end of an experiment unit volume of cells has changed to V and the final content of Cl is x, then the total Cl taken up by unit volume of cells, Vx, remains practically constant and is independent of the external concentration (Table II). When the external concentration of chloride falls very low, lysis of the erythrocyte occurs, but if the external osmotic pressure is sustained by the addition of glucose, lysis is prevented and it will be seen that the chloride concentration of the erythrocytes is fairly well maintained.

Table II.

(5 minutes' suspension at 23°.)

Ext. Cl	p_H	V	Cl	Vx	p_H	V	Cl	Vx
205	7	88	105	92	5·1	108	182	196
175	7	99	92	91	5·1	129	160	205
146	7	108	79	85	5·1	133	150	200
124	7	124	70	87	5·1	155	125	194
88	7	150	59	88	5·1	—	—	—
22*	—	—	—	—	5·1	88	176	155

* *Plus* glucose 9 %.

If the slowly penetrating glucose is replaced by a slowly penetrating anion like phosphate, the phosphate acts in the same way as the glucose; very little cell-base combines with phosphate, the bulk of it existing as chloride.

(3) *When erythrocytes are placed in a mixture of salt solutions the sum of all the anions entering the cell in five minutes is the same as the amount of chloride entering erythrocytes suspended for the same time in pure KCl solution of the same* p_H. This follows from the preceding observation. If the second anion B permeates more slowly than Cl, Cl enters to excess to satisfy the combining power of cell-base, but with the passage of time, more and more of B diffuses into the cell displacing chloride until true equilibrium is reached. Similarly, if B permeates the more readily, excess of B enters the cell and is subsequently displaced by Cl. In other words the anion distribution during a quasi-stationary state is used as an index of penetrating power.

If therefore at a given p_H, unit volume of cells takes up X m.eq. of Cl from pure KCl solution and if from a solution containing equivalent amounts of KCl and KB, cells take up Y m.eq. of Cl, then the cell content of B, Z m.eq., should equal $X - Y$ m.eq. and the concentration of B relative to Cl, R, may be expressed as Z/Y or $(X - Y)/Y$.

The preceding paragraph itself involves two assumptions:

(*a*) that at a given external p_H, a constant difference exists between the internal and external p_H, whatever the nature of the external salts may be;

(*b*) that at a given p_H inside the erythrocyte, the cell has the power of taking up constant amounts of anions whatever the nature of the diffusing salts.

Neither of these assumptions is strictly correct, but with regard to the second the following may be said. The rate of entry of a substance into a cell depends on its ability to penetrate the cell membrane and on the amount of the substance that the cell can take up. If B is an acid it can exist inside the cell as an anion, as an undissociated acid or as a complex combination with haemoglobin. It follows that if B has a low dissociation constant, or if it forms complexes with haemoglobin comparable with the formation of carbhaemoglobin by CO_2, then its concentration gradient will be relatively greater than that of Cl and it will tend to enter the erythrocyte in greater

amounts; but its power of penetrating the cell membrane will not necessarily be greater than that of Cl.

Now X equals the total anion capacity of cell-base expressed in terms of chloride equivalents; Y represents the amount of Cl combined with cell-base in the presence of anion B and $X-Y$ represents the amount of B combined with cell base also expressed in terms of chloride equivalents. Under these circumstances, it is thought that the value $(X-Y)/Y$ more truly indicates the relative permeation of B and Cl than Z/Y, for Z represents cell-B, dissociated, undissociated and complexly combined.

Further with regard to both the assumptions (a) and (b) it may be said that these approximate statements receive justification from Table III, where, with the exception of the phosphate-chloride system, total cell-anion (B and Cl) is nearly constant at each p_H level.

Table III.

(5 minutes' suspension at 23°.)
Ext. B = 87·5 m.eq.
Ext. Cl = 87·5 m.eq.

Ext. anion (B)	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.
Chloride	7	92	6	150	5·1	205
Iodide	7	98	6	159	5·1	211
Phosphate	7	87	6	133	5·1	182
Iodoacetate	7	92	6	160	5·1	212
Oxalate	7	86	6	146	5·1	198
Salicylate	7	96	6	149	5·1	—

It is thought that the low figure provided by the phosphate-chloride mixtures in acid ranges is due to some specific action of the anion on the cell membrane. Such decrease of permeation also invalidates assumption 3 and may be suspected where increased acidity causes a decrease of cell volume accompanied by agglutination.

It must be clearly understood that though the value $R=(X-Y)/Y$ approximately indicates the relative concentrations of B + Cl in erythrocytes it would only indicate the relative permeation of B + Cl if the cells were naturally chloride-free. But, in fact, R does not show the relative permeation of B and Cl because at the inception of any experiment erythrocytes have a natural chloride content of about 50 m.eq., and chloride therefore has an advantage over B, which is normally absent from the cell. If one were examining a system at equilibrium this initial Cl content would be unimportant, but since the comparison of anion permeation depends on the relative anion concentration before equilibrium has been reached, the initial Cl content is significant.

Although one cannot accurately compare the permeation of B relative to Cl one can however compare the ratios B/Cl, C/Cl, D/Cl and so obtain a more accurate idea of the relative permeations of B, C and D. Further, if the ratio B/Cl is equal to 1 or is greater than 1, it may be said that B permeates more readily than Cl, while if R is very much less than 1, one may infer that B permeates less readily.

It would, of course, be easy to remove chloride from erythrocytes by a preliminary soaking in potassium sulphate solution and to suspend the chloride-free cells in a mixture of KB and KCl, but it has been found that the permeability of cells twice suspended in salt solutions differs greatly from that of cells only once suspended, an observation which agrees with the experiments of Brinkman and Van Dam [1920].

Method.

Normal human blood was centrifuged in two tubes drawn out into sealed graduated and calibrated capillaries for thirty minutes at 4000 r.p.m. The volume of erythrocytes was noted and the cells in each tube then suspended in one hundred times their volume of the same test-fluid at 23°. This fluid usually contained 87·5 m.eq. of the test-anion (B) and 87·5 m.eq. of chloride. After 5 minutes the tubes were respun, the p_H of the clear fluid determined colorimetrically and the change in volume of the erythrocytes noted. The containing tube was carefully washed free of all trace of salts with distilled water, which was introduced with a capillary pipette down as far as the upper face of the erythrocyte column. The cells were then analysed. Sometimes it was necessary to examine the stability of anion distribution in erythrocytes, and under these circumstances the cells were suspended for periods varying from one to sixty minutes, and the relative anion contents were then compared.

It is clear that errors in reading the p_H of an unbuffered solution are much greater than if the solution is buffered. In fact, however, the external solution is never completely unbuffered, because a trace of bicarbonate diffuses into it from the suspended erythrocytes. To limit p_H errors as far as possible, two solutions of each indicator were used, one at each end of the corresponding p_H range. The method in the case of slightly alkaline KCl solution was as follows. A few drops of slightly acid phenol red were added as a layer on the surface of the clear supernatant fluid in one of the centrifuge-tubes, while to the fluid in the duplicate tube the slightly alkaline phenol red was added. As each indicator diffused downwards, its colour changed slightly, the orange solution becoming slightly redder and the red solution becoming slightly yellow. The weakest clearly perceptible colour in the supernatant fluid was matched with an inverted p_H colour-tube, comparison being made with a layer of suitable thickness in the tapering portion of the standard tube. It was thought that by these means a fair idea of p_H might be obtained.

Chloride was estimated by Claudius's method [1924]; phosphate by the method of Briggs [1922]; iodide and iodoacetate by the method of Kendall [quoted by Harington and Randall, 1929] and lactate by Mendel and Goldscheider's method [1925].

Salicylate was estimated by Benedict and Theis's colorimetric method [1924] slightly modified. Diazotisation was allowed to proceed for fifteen minutes. It was found desirable to prepare numerous standards so that the colour of the unknown did not differ by more than 20 % from that of the standard. Duplicate experiments on diffusion of salicylate into erythrocytes gave results which agreed within 10 %.

Oxalate was estimated by permanganate titration of the protein-free filtrate from cells treated with zinc hydroxide; a blank titration was done on normal cells.

Errors in the method are least marked in buffered solutions and are most evident in neutral unbuffered solutions. This arises from difficulties in reading p_H and from the relatively low chloride content to be estimated.

Errors in unbuffered solutions at p_H 7 are as follows:

Errors in tube volume	$\pm 0{\cdot}5$ %.
Errors in Cl estimation	$\pm 1{\cdot}5$ m.eq. in Y.
Errors in p_H reading	$\pm 0{\cdot}15\ p_H = \pm 7$ m.eq. in X.

Another possible source of error arises from differences in the haemoglobin content of unit volume of cells. In normal blood such differences are slight.

Table IV. *Summary of Results.*

Group	No.	Anion B	p_K		p_H 5·1					p_H 6·0					p_H 7·0				
					% of original vol.	Chloride m.eq.	B m.eq.	B/Cl calculated	B/Cl estimated	% of original vol.	Chloride m.eq.	B m.eq.	B/Cl calculated	B/Cl estimated	% of original vol.	Chloride m.eq.	B m.eq.	B/Cl estimated	B/Cl calculated
Inorganic	1	Chloride	—	—	129	205	—	1·00	—	114	150	—	1·00	—	98	92	—	1·00	—
	2	Phosphate	—	—	126	173	32	0·18	0·05	109	126	24	0·19	0·06	103	80	12	0·15	0·06
	3	Iodide	—	—	116	94	111	1·18	1·20	110	71	79	1·11	1·27	96	41	51	1·24	1·39
	4	Sulphate	—	—	122	126	79	0·63	—	115	112	38	0·34	—	103	76	16	0·21	—
	5	Nitrate	—	—	134	102	103	1·01	—	106	68	82	1·20	—	97	44	48	1·09	—
Organic	6	Thiocyanate*	—	—	134	84	94	1·12	—	103	60	90	1·50	—	93	44	48	1·09	—
ALIPHATIC Lower fatty acids	7	Formate	—	3·7	136	115	90	0·78	—	112	80	70	0·87	—	100	54	38	0·70	—
	8	Acetate	—	4·73	144	79	126	1·60	—	114	80	70	0·87	—	96	50	42	0·84	—
	9	Iodoacetate	—	3·15	136	80	125	1·56	1·75	113	81	69	0·85	1·00	98	52	40	0·77	0·77
	10	Propionate	—	4·8	140	82	123	1·50	—	112	80	70	0·87	—	100	54	38	0·70	—
	11	Butyrate	—	4·8	144	72	133	1·84	—	113	80	70	0·87	—	98	49	43	0·88	—
	12	Valerate	—	4·81	150	68	137	2·02	—	117	74	76	1·03	—	101	54	38	0·70	—
Hydroxy-aliphatic acids	13	Glycollate	—	3·84	130	117	88	0·75	—	110	94	56	0·60	—	97	64	28	0·44	—
	14	α-Hydroxypropionate	—	3·85	130	121	84	0·69	—	112	104	46	0·44	0·33	96	73	19	0·26	—
	15	β-Hydroxypropionate	—	4·61	136	95	110	1·16	—	108	94	56	0·60	—	96	66	26	0·39	—
	16	β-Hydroxybutyrate	—	4·52	137	92	113	1·23	—	113	87	63	0·72	—	101	67	25	0·37	—
	17	Glycerate	—	3·65	120	142	63	0·44	—	106	120	30	0·25	—	90	72	20	0·28	—
	18	Thiolacetate	—	3·55	128	70	135	1·92	—	110	68	82	1·20	—	104	60	32	0·53	—
Ketonic acids	19	Pyruvate	—	2·5	130	106	99	0·94	—	112	92	58	0·63	—	100	66	26	0·39	—
	20	Levulate	—	4·69	141	78	127	1·62	—	114	80	70	0·87	—	98	60	32	0·53	—
Dicarboxylic acids	21	Oxalate	1·42	4·39	105	28	177	6·32	5·60	106	71	79	1·11	1·0	97	48	44	0·92	0·81
	22	Malonate	2·8	5·68*	122	110	95	0·86	—	111	94	56	0·60	—	102	68	24	0·35	—
	23	Succinate	4·18	5·57	126	114	91	0·80	—	116	122	28	0·23	—	102	74	18	0·24	—
	24	Azelate*	4·62	5·6	115	72	106	1·47	—	108	74	76	1·03	—	102	75	17	0·23	—
	25	Maleate	1·93	6·58	114	110	95	0·86	—	115	106	44	0·41	—	106	75	17	0·23	—
	26	Fumarate	3·03	4·49	121	138	67	0·49	—	—	—	—	—	—	—	—	—	—	—
	27	Tartrate	3·0	4·39	126	164	41	0·25	—	—	—	—	—	—	103	79	13	0·16	—
Tricarboxylic acid	28	Citrate	—	—	—	—	—	—	—	116	126	24	0·19	—	—	—	—	—	—
AROMATIC	29	Benzoate*	—	4·2	123	72	106	1·48	—	106	60	90	1·50	—	96	49	43	0·88	—
	30	*o*-Hydroxybenzoate*	—	3·0	112	61	117	1·92	2·07	104	54	96	1·78	1·75	95	44	48	1·09	1·14
	31	*m*-Hydroxybenzoate	—	4·13	124	70	135	1·93	—	100	54	96	1·78	—	—	—	—	—	—
	32	*p*-Hydroxybenzoate	—	4·55	122	70	135	1·93	—	102	55	95	1·73	—	—	—	—	—	—
	33	*p*-Nitrobenzoate*	—	3·47	110	60	118	1·97	—	108	57	93	1·64	—	95	50	42	0·84	—
	34	*p*-Aminobenzoate	—	—	126	66	139	2·10	—	104	58	92	1·58	—	99	44	48	1·09	—
	35	α-Naphthoate*	—	4·7	110	56	149	2·67	—	102	48	102	2·13	—	93	44	48	1·09	—
	36	Phthalate	2·92	5·41	102	64	141	2·21	—	117	84	66	0·79	—	96	64	28	0·44	—
	37	Cinnamate*	—	4·46	108	59	119	2·02	—	—	—	—	—	—	—	—	—	—	—
	38	Mandelate	—	3·37	128	94	111	1·18	—	106	86	64	0·75	—	94	58	34	0·59	—
	39	Diphenylacetate	—	3·96	—	—	—	—	—	103	59	91	1·54	—	—	—	—	—	—
ALICYCLIC	40	Hexahydrobenzoate	—	4·88	—	—	—	—	—	118	72	78	1·08	—	98	48	44	0·92	—
	41	Quinate	—	3·54	118	156	49	0·31	—	107	123	27	0·22	—	95	75	17	0·23	—

* (At p_H 5·5.)

From a consideration of the values obtained in parallel experiments it is considered that where the ratio of B/Cl is in the neighbourhood of unity, differences exceeding 20 % are significant, but if the ratio is about 0·3, differences of permeation should not be assumed unless the ratios differ by 50 % or more.

The estimated ratio of B/Cl is shown in Table IV immediately after the calculated ratio. The organic anions in the Table are arranged in an aliphatic and an aromatic series, and these are subdivided into the simple anions and those containing additional polar groups, *e.g.* OH, COOH *etc.*

Inorganic anions and thiocyanate.

(Table IV.) The order of permeation is thiocyanate > iodide > nitrate, chloride > sulphate > phosphate.

Phosphate-chloride system. Solutions containing KH_2PO_4 and K_2HPO_4 (175 m.eq.) were mixed to give the required p_H, and an equal volume of KCl was added. It was thought better to use solutions of the same equivalence rather than the same osmotic pressure. Since phosphate may be univalent or bivalent within the p_H range investigated, it is not possible to determine accurately the content of cell-phosphate from the values for cell-phosphorus without knowing the p_H of the cell. But as over 80 % of phosphate is univalent between p_H 5 and 6·2 no serious error is involved if one assumes that phosphate is mainly univalent when the p_H of the external solution is between 5 and 6. In any case the figures for cell-phosphorus are so low as to make it clear that phosphate permeates very much more slowly than Cl (Table V).

Table V.

5 minutes at 23°.

p_H	% of original volume	Cell-Cl m.eq.	Cell-P mg.	P m.eq.	R
4·8	130	147	62	20	0·13
5·1	126	173	26	9	0·05
6·0	109	126	21	7	0·06

It will be noted that phosphate permeates at about the same rate between p_H 5·1 and 6, but at p_H 4·8, where some degree of haemolysis occurs, the phosphorus content rises sharply. It will also be seen that the permeation of phosphate is greatly depressed, while chloride enters to excess to satisfy the combining power of cell-base. Thus cells in pure phosphate solution containing 570 mg. P take up 235 mg. of P in 5 mins. at p_H 5·1, while if the phosphate is diluted with KCl so that its P content becomes 285 mg., cell-phosphorus does not diminish proportionately to 117 mg., for actually the cells take up only 26 mg. of P.

Effect of p_H *on phosphate permeation.* After 5 mins. suspension at 23° cells take up very little phosphate and the content is practically the same at p_H 5 and 6 (24 mg. P), but if the cells are immersed for 1 hour in phosphate-chloride solution at 33° (containing 87·5 m.eq. Cl and 285 mg. P) they take up 85 mg. P at p_H 5·1 and 215 mg. P at p_H 6. In other words if the exposure of erythrocytes to phosphate-chloride mixtures is prolonged, a certain impermeability develops at p_H 5 relative to p_H 6. It is thought that this may be due to a specific coagulation effect of phosphate at low p_H, which greatly decreases the permeation of phosphate itself, while leaving the permeation of the chloride ion relatively little affected.

The unequal distribution of phosphate and chloride inside erythrocytes is unstable, for the longer the cells are exposed to mixtures of phosphate and chloride, the more nearly does the distribution of these anions in the cells become proportional to that in the external solution.

Iodide-chloride (No. 3). Iodide was estimated directly and chloride, *Y*, was calculated indirectly and was also estimated directly from the total halide content of the protein-free filtrate of erythrocytes treated with sodium tungstate and sulphuric acid, silver nitrate and nitric acid being added and the solution titrated with thiocyanate, without previous boiling. The chloride content of the cells was also estimated by Claudius's method, for it was found that after prolonged boiling with $AgNO_3$, $K_2Mn_2O_8$ and HNO_3 the silver iodide was decomposed almost entirely, and a halide value was obtained which equalled the cell-chloride *plus* a small residue of iodide. Thus at p_H 6, iodide was 90 m.eq., giving a calculated Cl value of 60 m.eq. Total halide was estimated as 161 m.eq., giving an actual chloride value of 71 m.eq., which by the second method (acid incineration) was found to be 74 m.eq. At p_H 7, chloride was calculated at 35 m.eq. compared with estimated values of 41 and 39 m.eq.

It is clear that iodide attains a greater concentration in erythrocytes than does chloride, and this is the more remarkable when it is recalled that before iodide diffusion has begun chloride is already at its natural level of 50 m.eq. Moreover the unequal distribution is relatively stable for periods up to one hour; at p_H 5·9 cell-chloride and iodide were respectively 67 and 93 m.eq. while after 1 hour they were 69 and 96 m.eq.

Sulphate-chloride (No. 4), sulphate permeates less readily than chloride; this is least evident at p_H 5·1 and very marked at p_H 7.

Organic anions.

A large number of organic anions have been examined. Most of them have been standardised by titration, but in some cases salts of known purity were used. In the following, calculated (as opposed to estimated) values of B are shown in brackets.

All the organic anions appear to penetrate more readily at low p_H.

Lower fatty acids. This group, from acetate to valerate, appears to permeate more readily than chloride at p_H 5·1 and at about the same rate or very slightly more slowly between p_H 6 and 7. Table IV shows some indication that permeation becomes more rapid with increasing length of the carbon chain.

Formate (No. 7) is exceptional, for while between p_H 6 and 7 it has the same ratio as the acetate-valerate series, it permeates much more slowly at p_H 5·1.

Acetate-chloride (No. 8) (sodium acetate; KCl and HCl). At p_H 5·1, *R* is 1·60; at p_H 4·9, however, some haemolysis occurs, and *R* falls to 0·83. Presumably the damage to the cell membrane impairs its semipermeability so that the superior penetration of acetate is no longer in evidence.

Differences in permeation between acetate and chloride at p_H 5 and 6 are clearer if acetate and chloride are mixed in the proportion of 1 : 7 and cells are suspended for 5 and 60 mins. At p_H 6, at which acetate and chloride permeate at about the same rate, the distribution of acetate and chloride remains unaltered. At p_H 5·1, the superior permeation of acetate is shown by the low initial chloride at 5 mins. and its rapid increase during the subsequent 55 mins. (Table VI).

In order to confirm the superior permeation of acetate, an iodoacetate-chloride system was used, although the insertion of an iodine atom in the CH_3 group may well alter the permeation of the organic anion. However, the comparative similarity of the observed iodoacetate value and the calculated acetate

Table VI.

	External acetate 22 m.eq. p_H 5·1		External chloride 153 m.eq. p_H 6	
	Cell-chloride	Cell-acetate	Cell-chloride	Cell-acetate
5 mins.	107	(98)	129	(21)
60 mins.	170	(35)	131	(19)

figure lends support to the belief that the latter is approximately correct (Table IV, Nos. 8, 9).

Hydroxyaliphatic acids. The following were examined: glycollate, lactate, β-hydroxypropionate and β-hydroxybutyrate; these substances also permeate most rapidly at p_H 5·1 and more slowly at higher p_H. The addition of a hydroxyl group decreases penetration, and this decrease is much greater when the OH is in the α-position than when it is in the β-position. Indeed at p_H 5·1 the permeation of the β-hydroxy-anion is greater than that of Cl, but at p_H 7 it is probably less.

Lactate. The calculated values for lactate were checked by the veratrol colorimetric method. At p_H 5·05, lactate was calculated to be 89 m.eq. and estimated as 84 m.eq.; at p_H 5·7, lactate calculated was 44 m.eq. and estimated to be 38 m.eq.

The superior permeation of β-hydroxy- over α-hydroxy-anions is shown by a comparison of the B/Cl ratio at 5 and 60 mins. In the lactate mixture, the chloride which permeates first is displaced by the organic anion and decreases with time. In the β-hydroxypropionate mixture, chloride increases towards an equable distribution of anions.

Table VII.

(Ext. Cl 87·5 m.eq., Ext. B 87·5 m.eq.)

	Lactate p_H 5·1			β-Hydroxypropionate p_H 5·05		
	Cl	B	R	Cl	B	R
5 mins.	118	(87)	0·74	96	(114)	1·19
60 mins.	112	(93)	0·83	107	(103)	0·96

Glycerate (No. 17). This dihydroxy-acid permeates much more slowly than the preceding anion.

Thiolacetate (No. 18). The substitution of sulphur for the oxygen in the polar group increases permeation.

Ketonic acids. These acids like the hydroxy-acids also permeate less quickly than the corresponding fatty acids, and similarly when the carbonyl group is near the —COOH group permeation is slower than when it is further removed from the carboxyl group. Compare pyruvate with laevulate, which has the CO group in the γ-position (Nos. 19 and 20).

Amino-acids. The dissociation constants of these substances render them unsuitable for examination.

The dicarboxylates. These fall into two groups; oxalate which permeates more quickly than Cl, and the other dicarboxylates which on the whole permeate more slowly. The latter include the saturated malonate and succinate and the unsaturated maleate and fumarate. It also includes the hydroxy-anion tartrate.

All these acids have two dissociation constants, and they were adjusted to give a constant concentration of 87·5 m.eq. when mixed with an equal volume of KCl solution. Table IV shows that all these acids permeate more readily at low p_H; that they permeate less readily than oxalate; that succinate permeates more slowly than malonate, and that they all permeate more slowly than Cl at p_H 7;

that fumarate which is mainly bivalent at p_H 5·1 permeates less readily than maleate which is mainly univalent at this p_H. The table shows that the addition of hydroxyl or carboxyl groups to dicarboxylates makes it almost impossible for the anion to penetrate the cell membrane (*e.g.* tartrate (No. 27) and citrate (No. 28)). It must be noted however that azelate (No. 24), $(CH_2)_7(COOH)_2$ penetrates rapidly between p_H 5·5 and 6. It is thought that this may be correlated with its high solubility in organic solvents and its low dissociation.

Oxalate-chloride (No. 21). Oxalate was considered as a dibasic acid (p_K 1·42 and 4·39) at p_H 5·1. p_H was adjusted with oxalic or hydrochloric acid and in either case similar results were obtained.

Oxalate is unique in that it permeates more rapidly than chloride or any other anion at low p_H, and at p_H 7 it permeates more quickly than most anions and probably as quickly as chloride, for at p_H 7 cell-chloride remains practically unaltered while oxalate mounts from 0 to 39 m.eq.

The effect of length of exposure on the chloride-oxalate distribution at p_H 5·1 and 6·1 is as follows:

Table VIII.

	p_H 5·1			p_H 6·1		
Time	Cell-Cl	Cell-oxalate	R	Cell-Cl	Cell-oxalate	R
0 min.	50	0	0	50	0	0
¼ min.	23	131	5·7	38	104	2·7
1 min.	26	139	5·4	—	—	—
5 mins.	30	168	5·6	69	70	1·0
60 mins.	83	126	1·5	—	—	—

So rapidly does oxalate diffuse that in a few seconds its concentration is six times as great as that of chloride. With the passage of time however cell-Cl rises and oxalate falls so that equality is approached but is not nearly attained in one hour. At p_H 6 equality of distribution is reached in 5 mins., but even here the superior permeation of oxalate can be shown by exposing cells to oxalate-chloride solution for only a few seconds, when it will be found that oxalate has increased to 104 m.eq. while chloride has decreased to 38 m.eq.

While superior permeation of oxalate explains its high initial cell concentration followed by its subsequent decrease, it does not explain the corresponding changes in cell-chloride, which falls from 50 to 23 m.eq. in a few seconds and then rises at 1 hour to 83 m.eq. This can only be due to an actual displacement of chloride from the cell followed by its return into the cell again.

For this power of displacing chloride there seems to be no adequate explanation.

The oxalate-chloride system also illustrates the depression of chloride permeation by the more mobile anion. Cells in pure KCl at 175 m.eq. at p_H 5·1 take up 205 m.eq. Cl. In a mixture containing 87·5 m.eq. each of oxalate and chloride, the cells instead of taking up 102 m.eq. Cl actually lose 30 m.eq. and give a Cl content of about 20 m.eq. Even if cells are placed in a mixture containing 155 m.eq. Cl and only 22 m.eq. of oxalate at p_H 5·1, they take up only 58 m.eq. of chloride and as much as 115 m.eq. of oxalate in 5 mins.

A similar unequal distribution of oxalate and chloride (and also of acetate and chloride in the case of acetate-chloride mixtures) is obtained if freshly collected unheparinised blood is added directly to the salt mixtures.

It should be noted with regard to oxalate that it must be numbered among those anions which decrease permeation and cause shrinkage of cell volume at

low p_H. Thus in oxalate-chloride mixtures, cell volume at p_H 5·5 is 111 %; at p_H 5·1, 105 % and at p_H 4·5, 102 %, while the corresponding total anion contents are respectively 168, 198 and 167 m.eq.

Aromatic anions.

Benzoate permeates rather more readily than chloride, but the following anions, *o*-, *m*- and *p*-hydroxybenzoate, nitrobenzoate and *p*-aminobenzoate, though containing a carboxyl group and an additional polar group, all permeate more readily than benzoate itself. This superior permeation is most marked at low p_H.

Phthalate (No. 36), which contains two carboxyl groups, permeates rapidly at p_H 5·1 but at p_H 7 its entry is slower than that of any of the preceding anions and probably slower than that of chloride itself.

Naphthoate (No. 35), which contains two benzene rings, penetrates with great speed and can be qualitatively recovered from the erythrocyte. Experiments with naphthoate, nitrobenzoate and cinnamate were carried out at p_H 5·5, 6 and 7, and acidity was adjusted by the addition of phosphate solution to equal 25 m.eq. in the final mixture. In this way precipitation of the acid at p_H 5·5 was avoided while, owing to its slow permeation, effects due to phosphate itself could be neglected.

Salicylate-chloride. Salicylate was determined directly and indirectly. The salicylate-chloride values are shown in Table IV (No. 30). The unequal distribution of these anions is not entirely stable, but even after erythrocytes have been suspended in salt mixtures for 30 mins. at 33° and after haemolysis has occurred, it is very evident that salicylate is still present in a concentration which is much higher relatively than Cl (Table IX). This is most clearly seen when the external solution contains 153 m.eq. of chloride and only 22 m.eq. of salicylate (Table IX).

Table IX.

p_H 5·7.

Time	Temp.	External Cl	External salicylate	Cell-Cl	Cell-salicylate
½ min.	23°	153	22	56	91
5 mins.	23°	153	22	63	93
30 mins.	23°	153	22	82	68
60 mins.	33°	153	22	120	50

Three of the anions examined contained benzene rings attached to an aliphatic side-chain; of these cinnamate and diphenylacetate permeated quickly. But mandelate (No. 38) which contains a hydroxyl group in the aliphatic chain resembles the aliphatic anion glycollate in its penetrating power and not salicylate (No. 30) which contains the hydroxyl group attached to its benzene ring.

The permeation of the saturated alicyclic anions, hexahydrobenzoate and quinate, resembles that of the aliphatic acids, in that the former permeate quickly, while the latter hardly penetrates at all.

Discussion.

The view is generally held that the erythrocyte consists of a semipermeable envelope holding in its interior water, haemoglobin, salts and possibly lipin. The envelope contains lecithin, cholesterol, probably haemoglobin and also a stroma protein, isoelectric at p_H 5·5, described by Jorpes [1932].

Erythrocytes are impermeable to cations at $p_H < 8$, and this indicates that the cell membrane must be positively charged [Mond, 1928]. On the other hand, cataphoresis shows that the surface is negatively charged [Höber, 1904]. But as Mond remarks, the cell membrane may consist of two elements; an electronegative, giving the cell its cataphoretic properties, and a positively charged portion making the cell impermeable for cations.

The present investigation is concerned with the passage of anions through this complex envelope. The work of Henderson [1928] and of Van Slyke on electrolyte equilibria and the experiments of Höber [1910] on conductivity show that within the erythrocyte chloride is mainly ionised, and as it has been found that other anions quantitatively replace chloride, it may be presumed that these are also ionised. Further it has been found that erythrocytes take up anions from acid salt solutions, and as the process is accompanied by cell swelling, it seems probable that the bulk of cell-anion is distributed within the erythrocyte and not on its surface.

The difference of acid distribution within erythrocytes may depend on differing degrees of unionised complex formation or on differing ionic activities. In this case, one would expect the inequality of distribution to be stable. On the other hand unequal distribution may be due to differing rates of permeation through the cell membrane, in which event the inequality would be maximum at first and tend to diminish subsequently.

The only systems showing a stable inequality are iodide-chloride and thiocyanate-chloride, and the work of Hastings and Van Dyke [1931] on bromide-chloride distribution suggests that this may be due to iodide forming unionised compounds to a greater extent than chloride. In the case of salicylate-chloride, where the initial disparity in distribution diminishes to a fairly stable inequality, it is thought that both of the above mechanisms may be concerned. Most of the other systems show an unstable inequality which tends towards ultimate equality of anion distribution within the cell, and this is ascribed to differences of permeation through the cell membrane.

In view of the large size of some rapidly penetrating ions and the small size of others that permeate slowly, it seems unlikely that the pore theory of membrane permeability can hold with regard to the erythrocyte envelope, a probable alternative being that permeation rate depends on relative solubility in the cell membrane. It seems clear from Table IV that permeation does not depend on the solubility of anions in water, and the theory of Overton seems more applicable, namely that the penetration of substances into cells depends partly on their solubility in oily or lipoid substances. According to Jacobs [1924] the substances investigated by Overton fall into two groups—polar and non-polar. The latter (*e.g.* simple hydrocarbons) are unionised and soluble in organic solvents; these are said to penetrate cells rapidly; the former which contain groups like —OH, $—NH_2$ or —COOH tend to be dissociated and water-soluble; these penetrate cell membranes less readily, and the more numerous the polar groups, the more slowly does permeation occur. Attempts to support or refute these theories are necessarily inconclusive in the absence of data regarding the solubility of substances in the cell membrane. However, the permeation of substances into erythrocytes has been examined from the standpoints of their dissociation and their partition coefficients in an ether-water system.

Reference to Table IV shows that the simple aliphatic acids with one polar —COOH group permeate more rapidly than those with two polar groups, whether there be an —OH or an additional —COOH group, while substances like glycerate, tartrate and citrate (Nos. 17, 27 and 28) which have more than two

polar groups penetrate still more slowly. All these substances with numerous polar groups are more strongly dissociated and concentrate to greater extent than the corresponding fatty acids in the water phase of an ether-water system [Smith, 1921].

Overton suggests that organic substances permeate in the undissociated state, and it seems probable that the permeation of organic acids in general may depend on the dissociation constants of the acid and on its oil-solubility, varying according to the resultant of these two factors. Thus in the simple fatty acid group, the partition coefficient of formic acid is less than that of acetic acid, and the former is much more strongly dissociated, and it penetrates much more slowly at p_H 5·1, resembling the hydroxy-aliphatic acids in this. But at p_H 7, where both acids are well dissociated, the penetrations of formate and acetate are similar.

The members of the acetate-valerate series dissociate to an equal extent, but there is a marked increase in ether- and olive oil-solubility as the length of the carbon chain increases. The apparent increase of permeation however is only slight, but probably changes in the B/Cl ratio are masked by pre-existing chloride in erythrocytes.

The α-hydroxy-acids, glycollate and lactate are strongly dissociated and concentrate in the water phase; these substances penetrate much more slowly; the β-hydroxy-acids on the other hand are weak acids and, although their partitions are similar to those of the α-acids, their penetration is more rapid. The same applies to pyruvic and laevulic acids. In the former, the ketonic group is next the carboxyl; it is well dissociated and penetrates slowly; in the latter a γ-ketonic group is associated with low dissociation and more rapid penetration, although the partition coefficients of pyruvic and laevulic acids are similar.

Both the hydroxy- and keto-acids permeate more slowly than the corresponding fatty acids at higher p_H.

The dicarboxylic acids, malonic and succinic, are moderately well ionised at p_H 5·1, they concentrate in the water phase and their permeation is slow. But azelaic acid, $(CH_2)_7(COOH)_2$, on the other hand is less well dissociated and has an ether-water partition of 9·5. At p_H 5·1 it permeates rapidly, but at p_H 7, its permeation is slow. In this series, oxalate is exceptional, and it is not possible to correlate this with its power to precipitate calcium, for there is no detectable calcium in the erythrocyte, while even if calcium precipitation resulted in phase reversal of the cell membrane, there is no reason why oxalate should permeate more rapidly than the water-soluble anion, chloride or the ether-soluble organic acids.

In the case of the aromatic series, even complex substances like naphthoic and diphenylacetic acids permeate rapidly, and the presence of two polar groups as in the hydroxy-, amino- and nitro-benzoic acids does not seem to interfere with permeation. All these substances concentrate in the ether phase and attain a relatively high concentration in the oil of an olive oil-water system, and all quickly penetrate the erythrocyte membrane. It may be noted that while the presence of an —OH group in the benzene ring does not delay permeation, the substitution of —H by —OH in the aliphatic portion of phenylacetate greatly depresses permeation, which becomes comparable with that of the hydroxy-aliphatic acids: thus the permeation of mandelate is similar to that of lactate. It may be observed that the p_K, ether-water partition and permeation ratio at p_H 6 of salicylic acid are respectively 3·0, 5·0 and 1·78, while the corresponding data for mandelic acid are 3·37, 1·33 and 0·75.

The effect of p_H *on permeation.* The permeation of anions relatively to chloride decreases as p_H increases. This is due partly to the chloride which is normally present in erythrocytes, and the effect will be more apparent at high p_H, where the total anion taken up by the cells is less. But that the ratios obtaining at p_H 5·1 are not only due to this effect may be seen if the chloride figures at p_H 5·1 and 6 are compared. For not only does B increase as the total cell-anion increases, but in many cases cell-chloride remains unaltered and may even decrease, while in all cases the increase of B between p_H 6 and 5 is greater than the corresponding increase in chloride. This relative increase in permeation of anion B may be due to its being less dissociated at low p_H, but such an explanation could not apply to sulphate whose permeation relatively to chloride is also higher at p_H 5·1 than at 6. It might be thought that swelling of the cells would itself increase permeation by stretching the cell membrane, increase of permeation occurring *pari passu* with swelling. That this is not the case may be shown by suspending erythrocytes in hypertonic and hypotonic lactate-chloride solutions at p_H 7; in the latter solution, marked cell swelling occurs but the ratio of cell-lactate to chloride remains the same.

If one conceives the erythrocyte membrane as a negatively charged protein sieve whose holes are filled with lipoid material, then it follows that the restraining effect of the sieve protein meshwork on the permeation of anions will be decreased at low p_H. Further at low p_H the cell swells but the protein element will contract as it approaches its isoelectric point and as the total area of the cell membrane increases, it follows that the lipoid element must increase in area and decrease in thickness; this will differ from the effect of swelling resulting from hypotonicity where the protein and lipoid elements will be extended equally.

It is also possible that p_H may affect permeation directly through the lipoid portion. Condensed monomolecular films become expanded at low p_H [Adam, cited by Rideal, 1930] and such a change in the erythrocyte membrane may well alter its permeability. In this connection reference may be made to the high temperature coefficient of expansion of unimolecular fat films—a finding which is comparable in degree and range with the temperature coefficient of phosphate diffusion into erythrocytes [Maizels, 1932].

It is not suggested that lipoid-solubility is the only factor in, or even the direct means of, cell permeation, for adsorption, as well as solubility in organic solvents, is an expression of hydrophobe qualities. It is also possible that adsorption and permeation may occur at the protein-water interface of the cell envelope. Indeed it is likely that water-soluble substances and water itself may penetrate by this route.

Summary.

1. The order of permeation of inorganic ions into erythrocytes is (thiocyanate) > iodide > nitrate, chloride > sulphate > phosphate—a lyotropic series.

2. The permeation rate of anions relative to chloride increases with the external hydrogen ion concentration.

3. The presence of additional polar groups decreases the permeation of organic anions. This effect is less marked, if the polar group is not in the α-position to the carboxyl group, and it also becomes less marked with increasing length of the carbon chain.

4. Substances permeating rapidly are usually poorly dissociated, have a relatively high ether-water partition coefficient and are capillary-active.

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XLVI. DILATOMETRIC STUDIES IN THE PROTEOCLASTIC DEGRADATION OF PROTEINS.

I. TRYPTIC HYDROLYSIS.

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(*Received September 4th, 1933.*)

The enzymic digestion of complex substances like proteins is attended by a series of physical and chemical changes each of which proceeds at varying rates at different stages of the reaction. The viscosity change of the protein-protease system, for example, proceeds rapidly in the early stages of the reaction, while the changes in optical rotation or in the concentration of amino-groups during the same period are not correspondingly large. Each of these estimations is thus predominantly a measure of the particular type of change occurring during a definite phase of the reaction.

For obtaining an intimate knowledge of the mechanism of protein hydrolysis, it is necessary not only to utilise all the methods that are now available but also to invoke the aid of new technique which would either reveal changes not recorded by other means or independently confirm those indicated by other methods. The dilatometer, therefore, offers a new method of attack in the investigation of the hydrolytic cleavage of complex bodies in general and proteins in particular.

From the colloid chemical point of view, the degradation of the protein molecule can be looked upon as a process in which the degree of aggregation of the substrate is being diminished, the few colloidal micellae giving rise to several crystalloidal molecules varying in magnitude and composition. This process is accompanied by a disturbance in the water equilibrium of the system. From the chemical point of view the hydrolysis can be considered as a reaction involving the progressive release of amino- and carboxyl-groups.

Each of the above changes brings about a positive or negative volume change, the combined result of which is registered by the dilatometer. In order to resolve and apportion the net dilatometric change to its constituent physical and chemical reactions, the volume change specific to each of them has to be determined. These relationships can be ascertained by following the reaction during that phase of hydrolysis at which the change in question proceeds either predominantly or exclusively. When such a choice of phase is not possible, the object can be achieved by adopting such experimental conditions that one definite reaction proceeds to the practical exclusion of others. One of the possible ways of accomplishing this is to fractionate the enzyme complex into simpler constituents and investigate their action on the protein.

Galeotti [1911] has conducted a dilatometric study of the tryptic hydrolysis of peptone and milk. The reacting fluids are subject to changes in surface tension which would affect the change in the capillary column of the dilatometer. The tryptic digestion of milk would really constitute at least two definite systems, *viz.* milk-fat-lipase and caseinogen-trypsin, so that the observed volume change would not measure any one specific reaction.

While the present investigation was in progress, Rona and Fischgold [1933] have published a paper on the peptic digestion of ovalbumin and caseinogen in which they have tried to correlate the NH_2 release with volume change. A linear relationship was found to exist between the release of amino-groups and the dilatometric depression in the case of ovalbumin; such a relationship, however, was not obtained in the case of caseinogen. The experimental procedure adopted by Rona and his collaborators does not lend itself to a study of the interesting changes which occur during the first thirty minutes of the reaction. With the new type of dilatometer described in one of our earlier communications [1932] it is possible to investigate these changes accompanying an enzymic hydrolysis from its very commencement.

The present communication deals with the dilatometric study of the tryptic hydrolysis of caseinogen and gelatin. The reaction has been followed by an entirely independent method, *viz.* determination of amino-nitrogen by Van Slyke's method.

Materials. Hammerstein's "casein," Gold label gelatin and Pfansteil's trypsin (1 : 75) were the materials employed in the present investigation. All the reactions were conducted at p_H 7·7, employing Sørensen's phosphate buffer, and at a temperature of 30°. Caseinogen and gelatin digestions have been carried out at two different concentrations, and the experimental procedure adopted is similar to that fully described in our previous communication [1932].

Discussion.

The dilatometric fall is expressed in $mm.^3$ and the amino-nitrogen in mg. of nitrogen, and the data for each of the systems are graphically represented in Figs. 1 and 2, showing the course of hydrolysis of gelatin and caseinogen as followed by the dilatometer (Fig. 1) and the determinations of the amino-nitrogen (Fig. 2).

The general shape of the two corresponding sets of time-course curves, indicates that the kinetics of tryptic digestion can be followed in the dilatometer as well as by the determinations of amino-nitrogen. The dilatometric method offers a more elegant and less cumbersome technique for studying these reactions; by virtue of the ease and rapidity with which measurements can be conducted, the method possesses the additional advantage that, in the early stages, when enzymic reactions proceed rapidly, a greater number of readings could be obtained.

For the two systems investigated the relation between dilatometric depression and the corresponding increase in amino-nitrogen at different stages of the reaction (calculated from the graphs) is given in Fig. 3. The dilatometric fall in $mm.^3$ per millimol release of amino-nitrogen at different time intervals is given in Table I.

A close study of the Table and graphs will reveal the existence of an interesting series of relationships between dilatometric change and release of amino-groups. During the first thirty minutes there is no proportionality between volume change and amino-nitrogen, the dilatometer registering in

a given time a greater or smaller amount of change in proportion to the amino-nitrogen released during the same interval. It will be observed from Table I that the dilatometric depression per millimol release of amino-nitrogen in the case of caseinogen is greater in the initial stages, decreases with

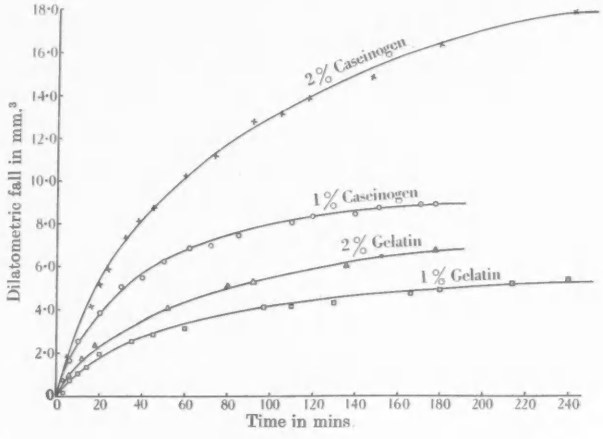

Fig. 1.

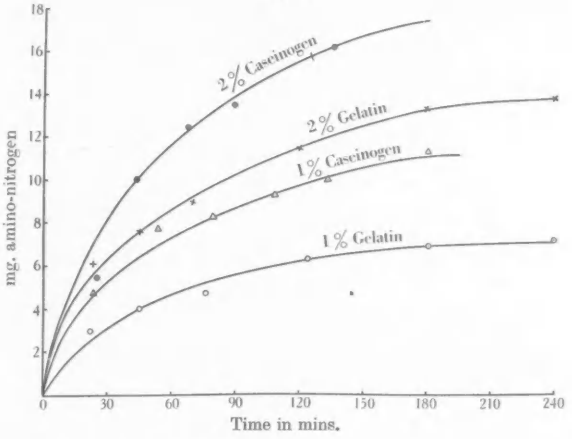

Fig. 2.

the progress of hydrolysis and attains practically a steady value after about 30–40 minutes. In the case of gelatin, however, the ratio increases to a steady value.

This fact is clearly borne out by the graphs (Fig. 3) where an attempt has been made to correlate volume changes with the formation of amino-nitrogen. It will be seen that a strictly linear relationship exists between them after about

Table I.

Time (mins.) ...	10	20	30	40	60	90	120
Dilatometric depression (mm.³) per millimol amino-N.							
Gelatin 1 %	8·8	9·3	9·7	9·8	9·8	9·7	9·7
Gelatin 2 %	6·1	6·5	6·6	6·9	7·1	7·0	7·1
Caseinogen 1 %	13·4	12·6	12·4	12·4	12·3	12·4	11·6
Caseinogen 2 %	16·0	14·8	14·0	13·5	12·8	12·4	12·4

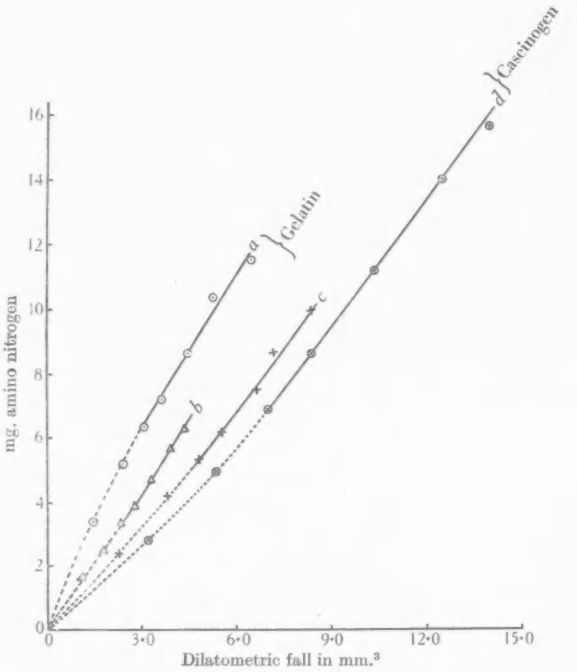

Fig. 3. *a*. 2 % gelatin. *b*. 1 % gelatin. *c*. 1 % caseinogen. *d*. 2 % caseinogen.

30 minutes in the cases both of gelatin and caseinogen. The magnitude of the initial change exclusively recorded by the dilatometer appears to be influenced by the relative concentrations of the enzyme and substrate and is closely connected with the process of liquefaction which generally precedes the rapid cleavage of proteins. A detailed study of this phenomenon is being made.

If the abnormal change occurring in the first stage of the reaction is ignored, a strict proportionality can be observed to exist between the dilatometric depression and the release of amino-nitrogen as shown by Fig. 3. The dilatometric depression per millimol release of amino-nitrogen, which is a constant for each protein, appears to be intimately associated with the structure and amino-acid make up of the protein. Gelatin has a constant of 8·7 while that of caseinogen is 10·8.

SUMMARY.

1. The course of the tryptic digestion of caseinogen and gelatin has been followed in the two-bulbed dilatometer which enables the reaction to be investigated from its very commencement.

2. The early stages of the reaction, during the first 30 or 40 minutes, are accompanied by changes registered by the dilatometer but not indicated by the determinations of amino-nitrogen. After this period, however, the dilatometric depression is proportional to the release of amino-nitrogen in the case of both the proteins.

3. The abnormality occurring in the initial stages of the digestion is closely connected with the process of liquefaction which generally precedes the rapid cleavage of proteins.

4. The dilatometric depression per millimol release of amino-nitrogen has been calculated and found to be 8·7 mm.^{3} in the case of gelatin and 10·8 in the case of caseinogen. This constant appears to be a function of the structure and amino-acid make up of the protein.

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XLVII. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

IV. THE EFFECT OF OVARIECTOMY AND OF INJECTIONS OF GONADAL AND ANTERIOR PITUITARY EXTRACTS ON URINARY CREATININE IN FEMALE RABBITS.

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(*Received September 26th, 1933.*)

The experiments on males detailed in the first three papers of this series [Schrire and Zwarenstein, 1932, 1, 2; 1933] were repeated on females. Creatine and creatinine were determined by Folin's method. The daily output of creatinine remained constant within comparatively narrow limits. The excretion of creatine, on the other hand, varied considerably. In all Tables, Rabbits Nos. 1, 2 and 3 are the normal animals and Nos. 4, 5, 6 and 7 the ovariectomised animals.

Ovariectomy.

In Table I the dates refer to the first of five 24-hour periods in each case. The mean and standard deviation are of the daily creatinine output in mg. given for each series of 24-hour periods. The weights of the animals at the beginning and at the end of the experiments are included.

Table I. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
	1	2	3	4	5	6	7
Weight, kg.	2·08	1·96	2·0	2·22	1·96	1·89	1·98
Date			A. Pre-ovariectomy periods.				
25. i. 32	65 ±4·6	—	76 ±1·9	72 ± 5·6	77 ±2·6	74 ±1·4	66 ±4·0
8. ii. 32	77 ±7·4	71 ±4·1	77 ±4·0	76 ± 3·6	74 ±6·0	72 ±4·2	79 ±2·1
15. ii. 32	70 ±3·6	67 ±1·9	74 ±3·9	76 ± 3·6	68 ±5·5	73 ±6·6	75 ±2·8
22. ii. 32	75 ±3·4	70 ±3·3	77 ±3·8	75 ± 7·2	68 ±5·3	74 ±4·0	75 ±1·0
			Nos. 4, 5, 6, 7 ovariectomised 9. iii. 32.				
			B. Post-ovariectomy periods.				
21. iii. 32	—	—	70 ±4·2	79 ± 3·6	—	67 ±3·9	76 ±3·2
18. iv. 32	—	—	71 ±3·9	82 ± 3·0	—	68 ±1·0	74 ±6·4
9. v. 32	—	—	57 ±4·6	74 ± 2·9	—	66 ±4·1	64 ±6·8
6. vi. 32	—	—	56 ±4·8	76 ± 5·5	—	62 ±4·5	67 ±2·8
4. vii. 32	—	—	74 ±6·3	76 ±10·5	—	68 ±3·7	75 ±8·7
13. viii. 32	—	78 ±5·6	78 ±5·1	67 ± 3·1	68 ±3·9	66 ±3·7	68 ±3·4
5. xii. 32	—	90 ±2·5	86 ±4·5	108 ± 8·5	99 ±8·2	98 ±3·5	89 ±6·3
19. xii. 32	92 ±3·7	98 ±7·0	88 ±4·8	104 ± 6·2	99 ±4·5	105 ±5·1	99 ±3·5
9. i. 33	84 ±2·2	92 ±2·7	87 ±3·6	102 ± 2·5	104 ±4·9	99 ±6·5	103 ±6·3
Weight, kg.	3·13	3·01	2·32	3·06	2·72	2·50	2·44

The data in Table I are summarised in Table II in the form of percentage increases in creatinine and body weight.

Table II.

	1	2	3	4	5	6	7
Creatinine	22	31	22	40	44	46	35
Weight	50	54	16	38	39	32	33

Inspection of the data shows that five months after ovariectomy the creatinine excretion was still normal. This result is in striking contrast to the effect of castration in males in which a post-castration rise in creatinine begins three to four months after removal of the testes.

Nine months after the date of ovariectomy an increased creatinine excretion occurred in all animals but the rise was significantly larger in the ovariectomised animals. The increases in creatinine output bore no constant relation to the increases in body weights. A comparison of the percentage figures for the normal and ovariectomised animals shows that the increase in creatinine excretion and gain in weight bore an inverse relation to each other for the two series of animals. The post-ovariectomy rise in creatinine was still maintained 12–14 months after the operation (Tables III and IV).

Table III. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
13. iii. 33	89	87	87	96	105	100	105
14. iii. 33	92	90	91	116	110	96	105
15. iii. 33	87	91	87	108	98	100	104
16. iii. 33	96	90	88	110	105	105	110
17. iii. 33	100	105	—	100	105	110	109
18. iii. 33	88	80	88	84	97	100	85
19. iii. 33	91	88	90	95	100	95	88
20. iii. 33	96	100	91	105	100	91	110
21. iii. 33	83	80	95	112	95	90	100
22. iii. 33	80	95	85	100	85	80	84
23. iii. 33	84	91	91	100	95	100	110
24. iii. 33	90	88	85	110	105	100	112
17. iii. 33	No injection	Ovary extract 10 cc.	No injection	Ovary suspension	Brain suspension	Brain extract 10 cc.	Ovary extract 10 cc.
21. iii. 33	Testis extract 20 cc.	Testis extract 20 cc.	No injection	Brain extract 10 cc.	Ovary extract 10 cc.	Testis extract 20 cc.	Testis extract 20 cc.

Table IV. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
24. iv. 33	95	90	88	105	116	110	105
25. iv. 33	100	95	81	116	110	110	116
26. iv. 33	88	91	90	116	105	100	116
27. iv. 33	89	88	90	105*	105*	99*	116*
28. iv. 33	91	100	89	95	89	70	110
29. iv. 33	95	95	91	89	95	105	110
30. iv. 33	91	88	85	100	96	100	110
1. v. 33	95	91	84	100	105	105	110

* 27. iv. 33 Nos. 4, 5 and 6 injected with saline suspension of ovaries.
No. 7 injected with saline suspension of brain.

Injection of saline suspensions of ovaries and of ovarian and testicular extracts.

Finely divided ovaries of cats or sheep and pigs were suspended in Locke's solution and injected subcutaneously. Ovarian and testicular extracts were prepared from sheep's or pig's ovaries or testes by the method of Moore, McGee and co-workers [McGee *et al.*, 1928; Moore *et al.*, 1930]. The final extract was taken up in olive oil before injection. Suspensions or extracts of brain were used as controls. 1 ml. of extract represents 93 g. testis, 58 g. ovary and 91 g. brain.

Injection of saline suspension of ovary into ovariectomised animals caused a decreased excretion of creatinine. In animal 6, Table IV, there was a fall to the pre-ovariectomy level. Both ovarian and testicular extracts caused a slight but probably not significant fall when injected into normal animals. Larger decreases occurred on injection into ovariectomised animals. In all cases brain suspension or extract had no effect.

Injection of anterior lobe extracts.

An extract of anterior lobe of sheep pituitary was prepared according to Bellerby's method [1933]. An extract of brain prepared by the same method served as a control. Both extracts were injected subcutaneously.

Table V. *Daily urinary creatinine.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
10. v. 33	96	88	85	99	105	102	107
11. v. 33	100	110	107	105	105	101	100
12. v. 33	93	111	82	105	105	116	112
13. v. 33	93	87	83	106	96	100	110
14. v. 33	84	86	91	105	100	105	108
15. v. 33	105	91	105	110	100	105	105
16. v. 33	88	91	88	110	100	98	116
17. v. 33	92	85	93	108	105	—	112
22. v. 33	88	84	88	110	116	—	105
23. v. 33	98	93	92	113	117	—	109
24. v. 33	95	110	108	100	105	—	105
25. v. 33	90	108	105	105	108	—	110
26. v. 33	88	88	91	105	116	—	116
27. v. 33	95	81	92	116	105	—	107

10. v. 33 Nos. 1 and 6 injected with 3·5 ml. brain extract.
Nos. 2, 3, 4 and 5 injected with 3·5 ml. anterior lobe extract.

14. v. 33 Nos. 1, 3 and 7 injected with 2·0 ml. anterior lobe extract.
Nos. 2 and 4 injected with 2·0 ml. brain extract.

23. v. 33 Nos. 2, 3, 4 and 5 injected with 4·0 ml. anterior lobe extract.
Nos. 1 and 7 injected with 4·0 ml. brain extract.

As in the same experiment with males, injection of anterior lobe extract into normal rabbits caused an increased excretion of creatinine, while injection into operated animals had no effect on the high creatinine output. Brain extract had no effect either in the normal or in the ovariectomised animals.

Discussion.

The results of ovariectomy and of injection of ovarian extracts affords evidence of the existence of an endocrine relationship between the ovaries and creatinine metabolism. The fact that both ovarian and testicular extracts have the same effect on the high creatinine level of ovariectomised animals suggests that the active principle in the ovarian extract is similar to, if not identical with, the active principle in the testicular extract.

Evidence presented in a previous paper [Schrire and Zwarenstein, 1933] suggests that the persistent increase in creatinine which occurs as the result of

castration is due primarily to hypertrophy of the anterior lobe of the pituitary. The results recorded above (Table V) indicate that the post-ovariectomy increase in creatinine is referable to the same factor.

Two outstanding differences emerge on comparing the effect of gonadectomy on creatinine excretion in the two sexes. The post-castration rise in creatinine commences 3 to 4 months after removal of the testes and in some cases sooner. In females the creatinine level is still normal 5 months after ovariectomy. It is unfortunate that owing to unavoidable circumstances no determinations could be carried out during the 6th–8th post-operative months. When determinations could again be resumed in the 9th post-operative month a definite increase in creatinine output was apparent.

In the ovariectomised animals the absolute individual rises in creatinine output above the values for the pre-periods were of the order of 41 %. Parallel normal controls showed an increase of about 25 % so that the true increase due to ovariectomy was actually about 16 %. Castration of males on the other hand led to a 25–50 % increase in the output of creatinine. Since in both sexes the effect on creatinine is primarily due to a pituitary hypertrophy, this difference is explicable on the assumption that functional hypertrophy of the anterior lobe is more pronounced in males than in females. This suggestion is supported by the observations of Hatai [1913] who found that the pituitary of castrated male rats was about 70 % heavier than that of spayed females, and by the results of Evans and Simpson [1929] who showed that the anterior lobe of the pituitary of gonadectomised male rats was more potent than that of gonadectomised females, as judged by the capacity of implants to produce precocious oestral changes in young females.

The excretion of creatine was too variable to allow of any certain conclusion regarding the effect of ovariectomy.

Summary.

1. Ovariectomy of adult female rabbits leads to a 16 % increase in the excretion of urinary creatinine commencing at least 5 months after the operation. The latent period is longer and the increase smaller than in castrated males.

2. Injection of saline suspension of ovary causes a fall in the high urinary creatinine of ovariectomy. Both ovarian and testicular extracts cause a slight fall when injected into normal animals. Larger and significant decreases occur on injection into ovariectomised animals.

3. Injection of extract of the anterior lobe of the pituitary leads to an increased excretion of urinary creatinine in normal females but has no effect on the high creatinine output of ovariectomised animals.

4. The results reported above afford evidence of an endocrine relationship between the ovaries and creatinine metabolism, this relationship being an indirect one through the anterior lobe of the pituitary.

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XLVIII. THE RELATIONSHIP OF THE METABOLIC NITROGEN OF THE FAECES TO BODY WEIGHT AND TO FOOD INTAKE FOR RATS.

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(*Received September 29th, 1933.*)

In the computation of biological values of proteins, investigators are divided in the manner of estimating the so-called "metabolic nitrogen of the faeces." Thomas [1909] and Martin and Robison [1922] thought this loss of body nitrogen to be constant, irrespective of the quantity of food ingested. Mitchell [1924] has considered it to be proportional to the amount of food dry matter consumed. Boas Fixsen and Jackson [1932], who favour the former viewpoint, make the following statement: "Mitchell [1924] argued that the amount of faecal nitrogen of endogenous origin excreted by a given animal was proportional to the weight of dry food ingested and that this ratio varied for different rats. In our experience, however, the results of several series of experiments on the same rat receiving nitrogen-free diets provide no evidence in support of such a relationship, at any rate where adult rats are concerned. On the contrary, in our work the same rat has shown an excretion of faecal nitrogen which is roughly constant in amount, in spite of large variations in the food intake...."

As these observations of Boas Fixsen are not in harmony with those made at this laboratory, the literature has been searched for evidence bearing upon the problem. A summary was made of all rat metabolism data relating to low-nitrogen diets that are available. This summary includes 1160 determinations of metabolic nitrogen (about one-third of which is from the author's unpublished data and the remainder from the literature) with rats of different body weights and consuming different amounts of food. The correlation of metabolic nitrogen and food consumed secured from these data is shown in Fig. 1. The regression line has a significant slope, which measures the rate of increase in metabolic nitrogen as more food is consumed. The rectilinearity of these data throughout most of their range seems apparent. The means of the arrays of the data when grouped follow a straight line except at the lowest and highest food intakes; the significance of these exceptions will be discussed later.

It cannot be stated, however, that all of the metabolic nitrogen excreted is proportional to the food consumed, since the intercept of the regression line on the y axis (zero food intake) is a significantly positive value. This part of the metabolic nitrogen obviously is not related to food intake.

There is, therefore, a constant fraction of the metabolic nitrogen, as well as a fraction which varies with food intake. If for any reason one fraction is much larger than the other, the impression may easily be created that the metabolic nitrogen is all of that fraction, either all constant or all proportional to food intake. Mitchell [1924; 1926] noted a relationship of metabolic nitrogen to body

[1] National Research Council Fellow in the Biological Sciences, 1931–33.

weight, but recognised with his small rats a much greater relationship to food intake. Boas Fixsen with large rats found "no evidence in support of such a relationship."

The correlation coefficient (r_{FM}) between food intake and metabolic nitrogen obtained from these 1160 observations is $0{\cdot}745 \pm 0{\cdot}009$. Correlation coefficients much nearer unity have been obtained by the author with data from individual rats[1]. The correlation coefficient (r_{WM}) between body weight and metabolic nitrogen is $0{\cdot}502 \pm 0{\cdot}015$, demonstrating that the relationship of metabolic nitrogen to body weight with these data is much less than the relationship of metabolic nitrogen to food consumption. To express adequately the relationship of metabolic nitrogen to food intake or body weight, irrespective of any relationship that may exist between body weight and food intake, it is necessary to make use of partial correlation coefficients. The partial correlation coefficient ($r_{FM.W}$) of food intake and metabolic nitrogen independent of body weight is $0{\cdot}744 \pm 0{\cdot}009$, while that ($r_{WM.F}$) of body weight and metabolic nitrogen independent of food intake, is $0{\cdot}499 \pm 0{\cdot}015$, indicating, as with simple correlation, that a closer relationship exists between food intake and metabolic nitrogen than between body weight and metabolic nitrogen.

The fact that the metabolic nitrogen in the faeces is related to body weight as well as to food intake, suggests that the constant fraction of the metabolic nitrogen, represented by the intercept of the regression line on the metabolic nitrogen axis in Fig. 1, varies with the body weight of the rat. Evidence confirming this suggestion may be deduced from these data. At the upper right-hand corner of Fig. 1, it will be noticed that the metabolic nitrogen values associated with the greatest food intakes, which were secured only with the larger rats, are all located above the regression line. Likewise, the points which are above the regression line at its lower end, and cause the data to depart from rectilinearity, were obtained again with the larger rats. In fact, the values obtained from larger rats tend to be above the regression line and those obtained from small rats below the regression line throughout the scale of food intake, the regression line representing the food intake-metabolic nitrogen relationship for the rat of average weight. Over two-thirds of the data from rats above average weight are located above the regression line, while the reverse is true with rats smaller than average.

There are 43 determinations of faecal nitrogen at a daily food intake of 9 g. that merit special study. The points referring to the larger rats are above the others, showing again that the larger the rat the greater is the constant fraction of the metabolic nitrogen. These 43 determinations with constant food intake show a correlation (r_{WM}) of $0{\cdot}830 \pm 0{\cdot}032$ between body weights and metabolic nitrogen values. The tendency for larger rats to have greater metabolic nitrogen excretions at any one level of food intake is evidenced also by the significantly positive partial correlation coefficient ($r_{WM.F} = 0{\cdot}499 \pm 0{\cdot}015$) between body weight and metabolic nitrogen.

Thus, it may be considered that the larger the rat the larger the constant fraction of the metabolic nitrogen, and assuming the same slope one may imagine a number of lines parallel with the regression line in Fig. 1, each line representing metabolic nitrogen as a function of food intake for one rat, the height of the line

[1] From data to be published elsewhere, the correlation coefficients between the food intake and the metabolic nitrogen of the same rat fed at from 10 to 19 levels of food intake were as follows for six different rats: $0{\cdot}981 \pm 0{\cdot}006$, $0{\cdot}971 \pm 0{\cdot}009$, $0{\cdot}993 \pm 0{\cdot}003$, $0{\cdot}964 \pm 0{\cdot}011$, $0{\cdot}937 \pm 0{\cdot}019$ and $0{\cdot}924 \pm 0{\cdot}023$.

varying with the weight of the rat. Hence, the data of Boas Fixsen, resulting from experiments with large rats (which consumed less food relative to body weight than small growing animals) show comparatively less change in the total metabolic nitrogen when food intake is varied and thus do not reveal the effect of food consumption on the metabolic nitrogen to the same extent as Mitchell's data with smaller rats, excreting correspondingly less of the constant fraction of the metabolic nitrogen and relatively more of the variable fraction related to the intake of food.

Another reason why Boas Fixsen failed to detect a relationship between metabolic faecal nitrogen and food intake pertains to her experimental technique, which does not appear to be sufficiently exacting. Maintaining a constant level of food intake during an experimental period of 4 days does not ensure regular excretion. Unless the same intake is continued for several days before the

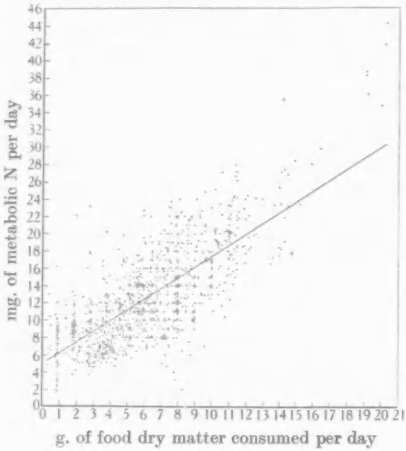

Fig. 1.

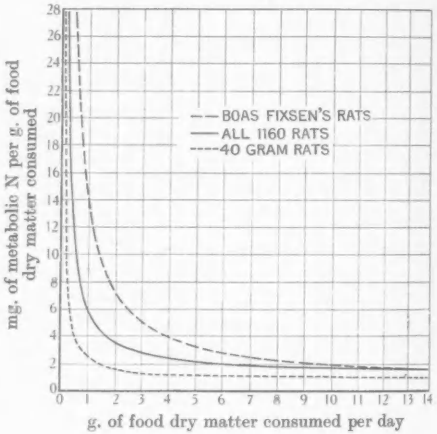
mg. of metabolic N per g. of food dry matter consumed
BOAS FIXSEN'S RATS
ALL 1160 RATS
40 GRAM RATS
g. of food dry matter consumed per day

Fig. 2.

collection period, there is a marked tendency for the amount of faecal dry matter collected at extreme levels of food intake, large or small, to regress toward the mean, *i.e.* toward the amount of faeces from the usual intakes of food prevailing before the experimental collection period. Longer collection periods would also diminish errors of this type. The determination of faecal nitrogen reveals little of quantitative value unless the amount of faeces analysed is excreted as a result of exactly that food intake to which it is ascribed. One cannot be sure of obtaining a quantitatively accurate sample of faecal dry matter in four-day periods without feeding a faeces marker. Distinct demarcation of faeces can be obtained by using alternately ferric oxide and chromic oxide to colour the first feeding of successive periods. Also, nitrogen-free diets, such as have been used by Boas Fixsen [1930], having little indigestible substance, do not favour regular defaecation. Furthermore, the slope of the regression line of metabolic nitrogen on food intake for such highly digestible diets is less than that of the regression line for those having more indigestible dry matter. Thus, the metabolic nitrogen of Boas Fixsen's rats increased on an average only 0·56 mg. per g. of dry food

consumed, while the average for all rats considered was an increase of 1·25 mg. of metabolic nitrogen per g. of food dry matter consumed.

Considering these factors of technique, involving the use of large rats, inaccurate demarcation of faeces, no preliminary periods[1], short collection periods and highly digestible diets, it is not surprising that the total metabolic nitrogen as measured should appear "roughly constant" for any one rat. Nevertheless, an examination of Fig. 1 reveals that the data of Boas Fixsen (indicated by circles) harmonise fairly well with the other 1103 observations. In fact, all of her data show a correlation coefficient between food intake and metabolic nitrogen of $0{\cdot}481 \pm 0{\cdot}069$, indicating a significant relationship for the pooled data which is scarcely suggested by the data from the individual rats which were fed at eight or fewer levels of food intake.

As a part of the metabolic nitrogen is constant and not proportional to food intake, it might be reasoned that to estimate the metabolic nitrogen in a period of protein feeding a regression equation (instead of the ratio introduced by Mitchell [1924]) should be derived from two levels of feeding of low-nitrogen food. The curves in Fig. 2[2] show the ratio "mg. of metabolic nitrogen per g. of food dry matter consumed" as a function of food intake. The ratio can be considered to be fairly constant and within the limits of experimental error for all but the lowest food intakes. The solid curve (derived from the data in Fig. 1) is for the "average" rat of the population. With larger rats the curve is to the right and above, its slope extending to greater food intakes. This indicates, of course, that with larger rats greater food intakes are required before the ratio becomes practically constant. The data from smaller rats produce a curve which is to the left and below, reaching a level at lower food intakes. Each curve approaches an asymptote for food intakes infinitely large, that is not influenced by the size of the rat. All curves approach infinity as the food intake approaches zero.

An increase in the amount of indigestible substance in the diet raises the position of the curve, but does not change its form. Hence, in determinations of the biological values of proteins it is important that the dry matter of the standardising low-nitrogen diet be of approximately the same digestibility as that of the protein-containing ration. The curves reveal that the ratio "mg. of metabolic nitrogen per g. of food," although not the most exact statement of the mathematical relationship between food intake and metabolic nitrogen, in practice lies well within the limits of experimental error when more than a certain minimum quantity of low-nitrogen food is ingested daily, and that it affords an appropriate means of computing the metabolic nitrogen in protein-feeding periods.

Conclusions.

The metabolic nitrogen in the faeces of rats consists of two fractions, one related to the body weight and the other to the intake of dry food.

The ratio of metabolic faecal nitrogen to the intake of dry food assumes a practically constant level for intakes of food above a certain minimum value, which seems to approximate to that required for maintenance of body weight in the case of the usual low-nitrogen diets used in protein evaluation work. Hence, in

[1] [Note by Editor.] The author is mistaken on this point [see Boas Fixsen, 1930, p. 1796].

[2] Considering that the regression line in Fig. 1 is described by the equation $y = a + bx$ in which y is the metabolic nitrogen, a the y-intercept, b the slope constant and x the food intake, the curves in Fig. 2 were fitted by the equation $y = \frac{a}{x} + b$, y being the ratio of metabolic nitrogen to food intake in the latter case, the other symbols having the same significance.

determinations of the biological values of proteins by Mitchell's technique, the ratio can logically be used in estimating the metabolic nitrogen in the faeces in the protein-feeding periods, if its limitations are born in mind. Furthermore, no better method of estimation appears to be available.

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XLIX. THE INFLUENCE OF ARSENATE AND ARSENITE ON THE ENZYMIC BREAKDOWN OF PHOSPHORIC ACID ESTERS.

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(*Received November 9th, 1933.*)

The experiments of Harden and Young [1906; 1911] showing that arsenate and arsenite ions accelerate the rate of liberation of inorganic phosphate from hexosediphosphate by the enzymes of yeast juice have led to much speculation as to the mechanism involved. Recent investigations of the effects of arsenate on the activity of the phosphatases and the glycolytic enzyme systems present in different kinds of biological material have yielded results which have been variously interpreted. All but the most recent of this work has been summarised by Harden [1932].

The view, originally expressed by Harden and Young, that the stimulating effect is exerted on the hexosephosphatase of yeast juice is open to criticism as the result of investigations in several laboratories. The experiments of Meyerhof [1927] with yeast and muscle, of Macfarlane [1930] and of Harden [1933] with yeast, and of Braunstein [1931] with rabbit erythrocytes support the conception that the effect is exercised not on the phosphatase but on the glycolytic system. This hypothesis is not considered by Morgulis and Pinto [1932] to be generally applicable in view of their results with rabbit and dog blood. Recently Braunstein and Lewitow [1932] have observed progressive diminution of the inorganic arsenate in mixtures of yeast, sugar, arsenate, toluene and water and they suggest that the accelerating effect of arsenate on fermentation and glycolysis depends on the formation of a labile hexosearsenate.

The present paper represents a study of the effects of arsenate and arsenite ions on the enzymic breakdown of phosphoric acid esters. The work was undertaken with the purpose of determining whether stimulation of phosphatase activity by these ions can be regarded as a general phenomenon or whether their effects on the rate of breakdown of phosphoric acid esters concern rather the mechanisms by which the organic portions of the esters are degraded by the accompanying glycolytic or similar enzymes. The materials chosen as the source of enzyme included four bacterial species, *Clostridium acetobutylicum* Weizmann, *Propionibacterium jensenii* van Niel, *Bacillus lactis aerogenes* and *Bacillus subtilis*, in addition to takadiastase, dried yeast, pig kidney and ox erythrocytes. Both sodium hexosediphosphate and sodium glycerophosphate were employed as substrates since the restricted use of the former in the investigation of mixtures containing both phosphatase and glycolytic systems might conceivably lead to erroneous conclusions. Furthermore, since the early work of Buchner [1903] and of Harden and Young [1911] showed that arsenite also accelerates the rate of fermentation by yeast juice, examination of the influence of this ion has been included in the present study. The method described by Pett [1933] for the

determination of inorganic phosphate in the presence of arsenate permits greater accuracy in the investigation of the effects of this ion in a wide range of concentrations than was hitherto possible.

Experimental.

Enzyme preparations.

Bacteria. Cl. acetobutylicum and *P. jensenii*, in the dried form, were prepared in the manner previously described [Pett and Wynne, 1933, 1]. *B. lactis aerogenes* was grown for 4 days in a sterilised medium containing 3 % glucose and 0·6 % peptone in diluted yeast extract. The organisms were centrifuged, washed once, dried and ground. *B. subtilis*, grown for 6 days in a similar medium, was treated in the same manner. The bacteria were used in the proportion of 0·1 g. dried organisms per 10 ml. enzyme digest.

Yeast. S. cerevisiae, isolated from baker's yeast, was grown in a medium containing 5 % glucose, 1 % peptone and suitable inorganic salts dissolved in diluted yeast extract. After 5 days the organisms were centrifuged, washed, dried and ground as usual; 0·05 g. dried cells was used in 10 ml. of digestion mixture. An extract of dried yeast was prepared by shaking 5 g. of the powder with 100 ml. of chloroform-saturated water for 24 hours. The mixture was then centrifuged and filtered; 1 ml. of the clear filtrate was used in 10 ml. digest.

Takaphosphatase. 20 g. of commercial takadiastase were suspended in 50 ml. of water, chloroform was added and the mixture was shaken for 15 hours. A small insoluble residue was removed; the solution contained a large amount of free phosphate which was eliminated by adding the equivalent amount of $MgCl_2$ and dilute NH_4OH until precipitation was just complete. Of the filtrate from this precipitate 1 ml. was used in 10 ml. of enzyme digest.

Mammalian kidney[1]. Fresh pig kidney was ground with twice its weight of water and allowed to stand for several hours. The mixture was filtered, and the filtrate was adjusted to p_H 6·0; after standing for 15 hours the solution was again filtered. The filtrate was adjusted to neutrality and stored in a refrigerator; 0·5 ml. of this solution was used in 10 ml. of digestion mixture.

Ox erythrocytes. The oxalated blood was treated in the manner described by Roche [1931]. Two ml. of the haemolysed solution were added to 0·5 ml. of buffer, 0·5 ml. of solution of arsenate or arsenite and 2 ml. of 2·5 % sodium glycerophosphate or of 1·25 % sodium hexosediphosphate.

Substrates.

The sodium glycerophosphate employed was obtained from The Eastman Kodak Company and consisted very largely of the β-ester. Sodium hexosediphosphate was prepared from the commercial calcium salt; the final preparation contained a small amount of monophosphate and a trace of inorganic phosphate.

General conditions.

The p_H optima of the phosphatases investigated are shown in Table I. The optimum for the yeast enzyme acting on hexosephosphate was reported by Kertesz [1930], for takaphosphatase on glycerophosphate by Kay and Lee [1931] and for kidney-phosphatase by Kay [1926]. The optima for the enzymes of the first two species of bacteria listed in the table have been reported by the present

[1] The authors are grateful to Mr G. A. McVicar for a supply of kidney-phosphatase prepared in this manner.

authors [1933, 1]. All the other recorded optima were determined as a part of the present investigation.

Table I. *Showing the* p_H *optima of various phosphatases.*

Enzyme source	Substrate: Hexosephosphate	Substrate: Glycerophosphate
Cl. acetobutylicum	6·0	5·1
P. jensenii	6·0	7·0
B. lactis aerogenes	5·8	5·5
B. subtilis	6·8	10·8
Sacc. cerevisiae	6·3	4·0
Takadiastase	4·2	4·0
Mammalian kidney	8·9	8·9
Ox blood cells	6·5	6·1

Potassium hydrogen phthalate buffers were used in the acid range and glycine buffers in the alkaline range; at p_H 7·0 no buffer was used. All solutions were adjusted to the desired p_H before mixing and the p_H of each mixture was determined after hydrolysis. No change greater than 0·3 p_H was observed.

Solutions of Na_3AsO_4 and of Na_2HAsO_3 having the following concentrations were prepared: M, $M/4$, $M/10$, $M/25$, $M/50$, $M/75$, $M/100$ and $M/200$. Of these solutions 1 ml. portions were added to 9 ml. of digestion mixture.

The total volume in each case was 10 ml., made up of 4 ml. of substrate (2·5 % glycerophosphate or 1·2 % hexosediphosphate), 4 ml. of buffer, 1 ml. of enzyme if liquid or 1 ml. of water together with the previously indicated weight of dried organisms, and 1 ml. of arsenate or arsenite solution. When other solutions, such as NaF, were added simultaneously simple adjustments of the volume of buffer were made, the concentration being kept constant. The mixtures were rocked in L-shaped tubes in a water-bath at 37·5°. At zero time and after 1 hour 2 ml. samples were added to 10 ml. portions of 5 % trichloroacetic acid solution. The precipitates were centrifuged and 10 ml. portions of the clear liquids were placed in 25 ml. volumetric flasks. If arsenate were present reduction was then effected in the manner described by Pett [1933]. Inorganic phosphate was determined by Fiske and Subbarow's method, using 3N acid; in several experiments duplicate results were obtained with Briggs's modification of the Bell and Doisy method.

Control tubes containing (*a*) enzyme, added ions and buffer, (*b*) substrate, added ions and buffer were incubated with the experimental tubes. The amount of inorganic P in the latter at zero time was subtracted from the 1-hour values, and from the figures so obtained was subtracted the sum of the increases in inorganic P in the two control tubes. Other tubes containing enzyme, buffer and substrate but no arsenic were included in all experiments. To the net amount of inorganic P (after allowing for the P released in suitable controls) liberated in such a digest containing a given enzyme preparation was assigned a value of 100. All the other results obtained with the same enzyme were compared with this standard value. The results are expressed in Table II.

Effect of fluoride.

In every instance of acceleration by arsenate or arsenite the acceleration could be lessened or removed by NaF in suitable concentration. This observation is complicated by the fact that fluoride retards the phosphatase activity of certain cells, such as yeast [Lipmann, 1928] and muscle [Lipmann, 1929]. However, fluoride up to $M/100$ concentration does not affect the hexosephosphatase

Table II. *Effects of arsenate and arsenite on the activity of various phosphatase preparations on glycerophosphate and hexosephosphate.*

Figures give the percentage hydrolysis under the influence of ions shown, as compared with the hydrolysis without added ions.

Conc. of ion in digest.	*Cl. acetobutylicum*		*P. jensenii*		*B. lactis aerogenes*		*B. subtilis*		Takadiastase		Yeast: Dried		Yeast: Aqueous extract		Pig kidney		Ox erythrocytes	
	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate	Glycero-phos-phate	Hexose-phos-phate
Arsenite:																		
M/2000	100	100	**100**	**100**	97	99	99	97	98	**113**	100	**100**	100	100	100	100	102	**108**
M/1000	100	100	**115**	**107**	90	97	98	96	101	**143**	100	**100**	100	100	100	100	100	**120**
M/750	101	100	**118**	**113**	—	—	97	94	—	—	100	—	—	—	100	103	—	—
M/500	100	97	**114**	**117**	87	96	95	91	100	**158**	100	**108**	100	100	100	102	100	**132**
M/250	98	95	**112**	**106**	84	94	95	88	103	**158**	95	**119**	100	98	97	105	100	**135**
M/100	90	93	**90**	**95**	76	93	95	83	103	**151**	94	**110**	95	96	95	106	91	**114**
M/40	—	—	—	—	—	—	—	—	97	**113**	92	**98**	90	—	—	—	90	**93**
M/10	—	—	—	—	—	—	—	—	—	—	—	—	—	95	92	92	—	—
Arsenate:																		
M/2000	100	100	**98**	**108**	101	99	93	98	100	**97**	98	**98**	100	100	98	100	103	**101**
M/1000	80	98	**117**	**122**	86	100	90	84	91	**141**	79	**115**	98	100	81	96	96	**115**
M/750	75	99	**123**	**132**	—	90	88	83	—	—	—	—	—	—	—	—	—	—
M/500	78	95	**116**	**136**	82	89	87	79	60	**167**	62	**161**	80	96	54	54	84	**130**
M/250	67	87	**92**	**207**	76	81	83	75	45	**162**	55	**153**	63	96	48	28	73	**144**
M/100	68	71	**49**	**265**	50	69	82	74	23	**155**	38	**132**	58	97	33	17	73	**188**
M/40	—	—	—	**306**	—	—	—	—	—	—	—	—	43	—	—	10	62	**170**
M/10	—	31	—	**305**	—	—	—	—	15	**106**	25	**124**	34	100	12	0	—	—
M	—	—	—	**9**	—	—	—	—	—	—	—	—	—	—	—	0	—	—

activity of *P. jensenii* [Pett and Wynne, 1933, 1]. This system is therefore well adapted for the investigation of the relation of fluoride to arsenate acceleration. Table III shows the results of an experiment of this kind.

Table III. *Diminution of the accelerating effect of arsenate by fluoride.*

Arsenate	Fluoride	mg. P released in 10 ml.
none	none	0·542
M/100	none	1·381
none	*M*/40	0·530
M/100	*M*/1000	1·368
M/100	*M*/400	1·287
M/100	*M*/200	1·207
M/100	*M*/100	1·123
M/100	*M*/40	0·873

The series can be carried no farther than *M*/40 NaF because higher concentrations affect the phosphate determination. The process used in reducing the arsenate has no effect on the recovery of phosphate in the presence of fluoride. It is evident that fluoride definitely reduces the arsenate acceleration.

A different type of experiment is of interest in this connection. The phosphorus reagents were added to a flask containing 2 ml. *M*/500 arsenate and to another containing 2 ml. *M*/500 arsenate and 2 ml. *M*/100 fluoride. After standing for 1 hour at 50° the flask containing fluoride developed only about 75 % of the intensity of colour in the other flask. Na_3AsO_4 does not yield a precipitate with NaF, even in a solution saturated with respect to both, so that the method by which arsenate is removed from action is not one of precipitation.

It was considered possible that the Na ions in the NaF solution appreciably depressed the dissociation of the Na_3AsO_4, but experiments with NaCl and Na_2SO_4 indicated that up to *M*/40 concentration these salts could not depress the arsenate acceleration to an extent equivalent to more than one-quarter of the fluoride effect.

While these facts do not fully explain the fluoride action they suggest that the fluoride ion forms a "complex ion" with the arsenate ion. Arsenate is thus converted into an ineffective form.

Discussion.

The results recorded in Table II lead to several observations. (*a*) The liberation of inorganic phosphate by the various enzyme preparations was not uniformly affected by arsenate and arsenite ions. The release of phosphate was accelerated in some instances, unaffected in others and definitely retarded in the remainder. If it be assumed that the phosphatases of the various preparations are identical in their mode of action on both substrates, an assumption which is not yet free from difficulties, the enzymes might be expected to respond similarly to the influence of arsenate and arsenite. Since they do not, it can be concluded that the influence of these ions is exercised not on the phosphatase but on some other mechanism which by its operation liberates inorganic phosphate. (*b*) In cases in which arsenate accelerated the reaction, arsenite had a similar though usually less marked effect; the optimum concentrations of the two ions were not always identical. (*c*) No stimulation occurred with an aqueous extract of dried yeast though the activity of the dried yeast preparation itself was stimulated by both arsenite and arsenate. (*d*) Acceleration with glycerophosphate as substrate was observed only with the *P. jensenii* preparation.

It is probable that all the enzyme preparations possessed in addition to phosphatase a glycolytic mechanism capable of breaking down, at least partially, the hexose portion of hexosediphosphate. However, in only four of the nine cases in which the influence of arsenate on the degradation of this ester was studied was there any evidence of acceleration. It is apparent therefore that, in addition to having no consistent effect on the hydrolytic activity of the various phosphatases, the arsenate ion has no universally demonstrable accelerating influence on the activity of the glycolytic enzyme systems. This assumes that, under the conditions of the experiments, accelerated breakdown of the organic component of the ester would lead incidentally to accelerated accumulation of free phosphate.

The increase in inorganic phosphate as the result of the action of yeast, kidney, takadiastase, erythrocyte and *Cl. acetobutylicum* preparations on glycerophosphate can be regarded as proof of the presence of a true phosphatase because it is probable that none of these preparations is able to attack the glycerol portion of the ester. But no stimulation by arsenate or arsenite occurred with any of these glycerophosphatases. On the other hand all the other bacteria used can metabolise glycerol; it has been shown, furthermore, that *P. jensenii* [Pett and Wynne, 1933, 2] and *B. lactis aerogenes* (unpublished work of the authors) can, after drying, partially break down the glycerol residue of the ester. However, in the case of only one organism, *P. jensenii*, did arsenate or arsenite exert any accelerating influence on the rate of glycerophosphate breakdown.

A satisfactory interpretation of these results is not yet possible. Although it can be concluded that arsenates and arsenites do not accelerate the activity of phosphatases in general, the lack of uniformity of the effects of these ions on the activity of the various preparations, as measured by phosphate liberation, is somewhat confusing. The evidence supports the hypothesis that any observed accelerating effect is due to a stimulation of the enzyme system responsible for the degradation of the organic part of the ester. Furthermore the specificity observed suggests that the influence is exercised at some particular stage of this breakdown, common to some but not all of the preparations used. Further speculation must be supported by more complete experimental evidence.

In this connection it is of interest to observe that, although the aqueous extract of dried yeast possessed an active phosphatase which was not stimulated by arsenate or arsenite, the dried yeast itself responded to the influence of both ions. When tested in a 1 % solution of sodium hexosediphosphate in microfermentation tubes the yeast extract failed to cause the liberation of gas. It is evident that the complete zymase system was not present in the extract: possibly this deficiency was reflected in the failure of arsenate and arsenite to cause stimulation.

The experiments using cytolysed ox erythrocytes are of interest in view of the recent findings of Morgulis and Pinto [1932] and of Braunstein [1931]. The latter was unable to demonstrate arsenate stimulation of the phosphatase activity of rabbit serum and erythrocytes on hexosediphosphate, but in experiments in which the esters of the cells constituted the substrates for the accompanying enzymes he obtained results which were interpreted as indicating activation of the glycolytic mechanism by arsenate. On the other hand Morgulis and Pinto [1932] do not regard such stimulation of glycolytic systems as a general phenomenon because concentrations of arsenate which definitely accelerated the rate of glycolysis in mixtures containing rabbit blood preparations caused retardation of glycolysis using dog blood; in all cases the liberation of inorganic phosphates was accelerated. They therefore concluded that the increased rate of phosphate liberation in blood-glycolysing systems under the

influence of arsenate is due to a stimulation of the blood-phosphatases. In the case of ox erythrocytes, in the present work, arsenate acceleration of the rate of liberation of inorganic phosphate was observed with hexosediphosphate as substrate but not with glycerophosphate. If the stimulation were exercised on the phosphatase mechanism one would have expected acceleration of the hydrolysis of both substrates to occur. The evidence strongly suggests that in the case of ox erythrocytes the arsenate effect is exercised on the glycolytic system rather than on the phosphatase.

Summary.

The rate of liberation of inorganic phosphate from glycerophosphate and hexosediphosphate through the action of several enzyme preparations was studied in relation to the influence of varying concentrations of arsenate and arsenite ions. Preparations of the following were employed: *Cl. acetobutylicum*, *P. jensenii*, *B. subtilis*, *B. lactis aerogenes*, yeast, takadiastase, pig kidney and ox erythrocytes.

No evidence was obtained to show that the activity of phosphatase systems in general is accelerated by arsenate or arsenite. The observations support the hypothesis that the accelerated enzymic liberation of phosphate under the influence of these ions is due to an effect exercised at some stage of the breakdown of the organic portion of the phosphoric acid ester.

The diminution of the stimulating effect of arsenate by fluoride was investigated and the suggestion is advanced that this may be due to the formation of a complex ion, thus rendering a portion of the arsenate ineffective.

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L. EFFECT OF ULTRA-VIOLET RADIATION AND HEAT UPON PROTEIN SOLUTIONS OF LOW CONCENTRATIONS.

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(*Received December 16th, 1933.*)

Protein solutions of acid or alkaline reaction show a marked increase of absorptive power in the ultra-violet spectrum after prolonged irradiation with the light of a quartz mercury lamp [Spiegel-Adolf and Krumpel, 1927]. The shape of the absorption curve is not altered if the irradiation be carried out in ordinary air, the irradiated product in this case behaving like the original protein at higher concentration [Hausmann and Spiegel-Adolf, 1927; Spiegel-Adolf, 1928]. The protein irradiated under the same conditions but without addition of electrolytes coagulates spontaneously nearly completely, as has been shown in a previous paper [Spiegel-Adolf, 1927]. Becker and Szendrö [1931] working on highly diluted protein solutions (1·5–2·5 mg./100 ml.) found on the other hand that irradiation, especially in presence of oxygen, seemed to destroy the proteins. The degradation products though showing an increased absorption for ultra-violet radiation had lost the absorption band characteristic for proteins and their normal derivates. In order to explain this apparent discrepancy new experiments were started on protein solutions of different concentrations.

In a first series of investigations measurements of the refraction have been undertaken in crder to detect traces of cleavage of the protein irradiated under conditions comparable with those employed by Spiegel-Adolf and Krumpel [1927]. Boiled samples of uncoagulable protein mixtures were studied similarly, since Spiegel-Adolf and Krumpel [1929] have shown that such boiled protein solutions retain their original absorptive power. Changes of the refraction of proteins after denaturation by heat and strong acid have been reported by Obermayer and Pick [1905].

As in the proposed experiments little or no heat change was to be expected and only the effects of heat and light of short wave-length were to be compared, an interferometer (Zeiss-Löwe) was used. Since the interferometric value of a 0·1–0·05 % pseudoglobulin solution attained a stable value after 2 hours of irradiation with the light of a quartz mercury lamp (Hanovia, 220 v. d.c.) and the protein had at the same time practically disappeared from the solution, the irradiation time was limited in this series to 2 hours, the distance of 40 cm. making cooling unnecessary. The protein solutions used were pseudoglobulin and serum-albumin separated from horse serum by fractionation with $(NH_4)_2SO_4$, purified repeatedly by refractionation, dialysis and electrodialysis. The stock solution of the pseudoglobulin was 5 %, the c_H was $1{\cdot}40 \times 10^{-7}$, the conductance $\kappa = 1{\cdot}11 \times 10^{-5}$. The 2 % solution of serum-albumin had c_H $1{\cdot}17 \times 10^{-5}$, a conductance $\kappa = 9 \times 10^{-6}$. The amounts of HCl or KOH added to the protein solutions were within the range of those used in the previous communications.

As in former experiments the heating of the solutions in a water-bath under reflux was limited to 15 minutes. The results are summarised in Table I A and B. The figures are interferometric units, a 20 mm. cell being used.

Table I.

A. Serum-albumin.

(Final concentration in all samples 0·2 %.)

		Native	Boiled	Irradiated	Boiled and irradiated	Irradiated and boiled
0·001 *N* KOH	Obs.	325	333	331	326	331
	Calc.	330				
0·005 *N* KOH	Obs.	375	379	379	380	410
	Calc.	381				
0·001 *N* HCl	Obs.	320	322	326	329	350
	Calc.	326				
0·005 *N* HCl	Obs.	356	356	356	382	393
	Calc.	362				

B. Pseudoglobulin.

(Final concentration in all samples 0·1 %.)

0·001 *N* KOH	Obs.	196	198	199	203	199
	Calc.	201				
0·005 *N* KOH	Obs.	245	249	245	248	249
	Calc.	252				
0·001 *N* HCl	Obs.	194	196	199	196	203
	Calc.	198				
0·005 *N* HCl	Obs.	226	231	228	231	231
	Calc.	235				

Additions of acid or alkali to serum-albumin and pseudoglobulin give lower values than those calculated. These findings agree with those of Thomas and Mayer [1928], whose interpretation seems applicable to all kinds of proteins. The changes in refraction after irradiating or boiling are negligible; they do not exceed 3 % of the interferometric value of the native protein but seem to be outside the limit of error of the method. The influence of heating is most marked in alkaline solutions, *i.e.* under conditions favouring maximum hydrolysis, while irradiation shows its effects most clearly in an acid medium. Nevertheless in most cases there are no differences between the effects of heat and irradiation and when such differences do occur they are so small as to give no support to the suggestions of Becker and Szendrö [1931]. When heating precedes the irradiation the changes if any are much smaller [*cf.* Spiegèl-Adolf, 1929].

In a further series of experiments the behaviour of proteins submitted to heat or irradiation at various concentrations without additions of electrolytes was studied. The irradiation and the heating were done in the manner already described, the fluids after filtration through Schleicher and Schüll filter-paper No. 590 were tested for their refraction, protein-content and gold sol reaction.

The results are summarised in Table II A and B.

These results show that both kinds of denaturation are sufficient to precipitate the total protein; only in the most concentrated samples can traces of the latter be detected after irradiation for 2 hours at 40 cm. distance; these disappear on further exposure. Though especially in the highest degree of dilution denaturation seemed to cause but a faint opacity of the solutions it was always possible to get a clear filtrate by using the above-mentioned filter.

Interferometric measurements of the filtrates indicate that not all refractive material has been precipitated with the protein. This may mean that the interferometric method is more sensitive in detecting protein than flocculation by

Table II.

A. Serum-albumin.

Concentration %		Heat				Irradiation			
			IU	SSSR	GSR		IU	SSSR	GSR
0·16	*a*		307	+	+		307	+	+
	p	Coagulation	19	–	–	Coagulation	37	trace	+ + +
0·08	*a*		144	+	+		144	+	+
	p	Coagulation	13	–	–	Coagulation	22	–	+ + +
0·04	*a*		73	+	+		73	+	+
	p	Coagulation	7	–	–	Coagulation	21	–	+ + +
0·02	*a*				–				
	p				+				
0·016	*a*		27	+	–		27	+	–
	p	Coagulation	4	–	+	Coagulation	13	–	+ + +
0·004	*a*				–				
	p				–				
0·0032	*a*		6	+	–		6	+	–
	p	Opalescence but filtrable	5	–	–	Clear	8	–	+ + +

B. Pseudoglobulin.

0·2	*a*		374	+	+		374	+ +	+
	p	Coagulation	20	–	–	Precipitation	44	trace	+
					R_V				
0·1	*a*		193	+	+		193	+	+
							28	–	+
	p	Coagulation	8	–	+	Precipitation	43	–	+
0·05	*a*		91	+	+		91	+	+
	p	Coagulation	5	–	+	Precipitation	42	trace	+
0·02	*a*		48	+	+		48	+	+
	p	Coagulation	4	–	–	Precipitation	19	–	–
0·004	*a*		10	+	+		10	+	+
	p	Coagulation	0	–	–	Distinct opalescence	8	–	+
0·002	*a*		5	+	–		6	trace	–
	p	Coagulation	2	–	–	Doubtful opalescence	7	–	–

IU = Interferometric units in 20 mm. cell.
a = before; *p* = after.
SSSR = Reaction with sulphosalicylic acid.
GSR = Reaction with colloidal gold.
R_V = Red with trace of violet.

sulphosalicylic acid or that precipitation of the protein is accompanied by some cleavage, or finally that the solutions contain something else beside protein. It does not seem possible to exclude any of these possibilities altogether. But the fact that only in the case of heated proteins does the refraction of the filtrates decrease practically to zero with increasing dilution, while in the case of irradiation it reaches finally the refraction value of the original solution, seems to indicate that irradiation of proteins causes not only denaturation but also cleavage. This supposition agrees with the changes in odour and colour.

Further evidence of the cleavage of proteins submitted to irradiation is afforded by the gold sol reaction. This is more sensitive for pseudoglobulin than for serum-albumin but does not reach the sensitiveness of the sulphosalicylic acid test. Colloidal gold, however, can be used besides for the classification of proteoses, and, according to the explanation of the clinical gold sol tests, is able to reveal pathological changes in proteins where other methods fail.

Table II A and B shows that in the cases of heated proteins with the exception of serum-albumin in a range between 0·02 and 0·01 % the filtrates do not show any gold sol reactions. This confirms the results of Ogiu and Pauli [1932], who made similar observations, and the fact that the filtrates were practically free from protein. But the filtrates of irradiated proteins even when they show no trace of proteins with sulphosalicylic acid give a strong gold sol reaction. The type of the gold sol reaction is somewhat different from the two types of gold sol flocculation by proteins described by Ogiu and Pauli [1932], the change in the present case being directly from pink to blue and white.

The finding of a substance giving a positive gold sol reaction seems to confirm the results of Becker and Szendrö [1931] as to the partial splitting of the protein by irradiation. But the formation of this particular proteose depends upon the intensity and perhaps upon the wave-length of the radiation—the protein solutions themselves acting as selective filters, whose absorption is maximum between 292·7 and 261 $m\mu$. If for instance a 0·2 % serum-albumin solution is not filtered after the first hour of irradiation when it is nearly opaque but is irradiated for another hour, then the sulphosalicylic acid test becomes negative, but the gold sol reaction becomes negative too. If a solution of 0·5 % pseudoglobulin is irradiated in the usual way, after 4 hours it reaches a stage at which the sulphosalicylic acid test is still positive, but the gold sol reaction due to the protein content of the solution has already disappeared. Two more hours of irradiation cause total disappearance of the protein reaction, while the gold sol reaction becomes strongly positive. Nevertheless the solid content of the filtrate is only 1/10 of the initial protein content, and the end absorption starting at 230 $m\mu$ cannot account for the increased absorption of irradiated protein solutions containing acid or alkali within a range of 400–267·5 $m\mu$.

Finally experiments were performed under conditions similar to those used by Spiegel-Adolf and Krumpel [1927]. 0·5 % solutions of serum-albumin and pseudoglobulin containing 0·005 N HCl or KOH were irradiated during $5\frac{1}{2}$–6 hours at a distance of 30–40 cm. from the light source. The solutions were protected against evaporation by cork stoppers fitting tightly into the quartz test-tubes. (Controls of pure protein solutions were used in order to ascertain the effect of irradiation upon them.) After the exposure the solutions were exactly neutralised. They still contained traces of heat-coagulable proteins proving the existence of small amounts of undenatured protein. After renewed filtration the solutions showed a slight reaction with sulphosalicylic acid but no gold sol reaction. This was taken to show that the effect of irradiation depends greatly on the original protein content of the solution and on the amount of acid or alkali, as these determine the initial protein concentration through the entire experiment. The different kinds of cleavage caused in protein solutions of varying concentration or electrolyte content by irradiation may explain the discrepancy of the spectrographic findings of different authors which have been the starting-

Table III.

	Filtration	Period of irradiation hrs.	Electrolytes	Gold sol reaction
0·2 % serum-albumin	Filtration after 1st hour	2	None	+
0·2 % serum-albumin	No filtration	2	None	–
0·5 % pseudoglobulin	Filtration	4	None	–
0·5 % pseudoglobulin	Filtration	6	None	+
0·5 % serum-albumin or pseudoglobulin	Filtration	$5\frac{1}{2}$–6	0·005 N HCl or KOH	– (after neutralisation)

point of this paper. (The above-mentioned experiments are summarised in Table III.)

Becker and Szendrö [1931] have made elaborate investigations of the nitrogen content and certain chemical properties of the diluted protein solutions after irradiation. It seemed desirable to extend the investigation in a more physico-chemical direction, particularly concerning the relations to other proteoses of the substance giving a positive gold sol reaction.

In order to get solutions of this substance of higher concentrations 0·1 % solutions of serum-albumin and pseudoglobulin were irradiated in flat dishes at 15 cm. distance directly under the tube of the quartz mercury arc lamp; the surface of the dish was about 50 cm.^{2}, the fluid layer having a depth of 1 cm. After 6 hours of irradiation more than half of the total protein content, *i.e.* 0·026–0·03 g. of the initial 0·05 g. was precipitated; the fluid was then concentrated to 1/10 vol. on the boiling water-bath.

The solution was clear with a marked yellow tinge, the solid content was in several samples between 0·21 and 0·27 %, the specific conductivity was $3{\cdot}53 \times 10^{-4}$ ohms^{-1}, the c_{H} $6{\cdot}22 \times 10^{-6}$. The latter was measured with a hydrogen electrode, the whole outfit having been carefully checked before and after the determination. The absorptive power in ultra-violet radiation was different from that of protein, being, for the same solid content, rather higher than those of serum-albumin or pseudoglobulin. Using 5 mm. quartz cells a 0·4 % solution of serum-albumin absorbed all light from wave-length 245 $m\mu$, while the absorption of the filtrate from the irradiated sample started approximately at 320 $m\mu$ (Table IV).

Table IV. *Extreme non-absorbed wave-lengths.*

Concentration %	Serum-albumin $m\mu$	Filtrate of irradiated serum-albumin $m\mu$	Proteose of Witte peptone $m\mu$
0·4	245	320	—
0·2	235	275	245
0·1	231	240	—

Pseudoglobulin shows a similar behaviour. But contrary to the findings of Spiegel-Adolf and Krumpel [1927] for concentrated protein solutions irradiated in the presence of acid or alkali, the filtrate did not give any special absorption band beside the end absorption, confirming in this respect the findings of Becker and Szendrö [1931].

The filtrate gave a positive biuret reaction with a slight red-violet tinge and positive reactions with Millon's reagent and trichloroacetic acid, while the test with sulphosalicylic acid was negative and no H_2S could be traced after heating with alkali. Alcohol causes a slight opalescence when its concentration reaches 80 %. The filtrate shows a slight opalescence when saturated with ammonium sulphate but no change on half saturation. If the test is made according to the method of Nonne-Appelt-Schumm for spinal fluid it is negative or doubtful.

The filtrate flocculates gold sol in concentrations equivalent approximately to 0·0005 mg. in 1 ml., the derivatives of serum-albumin and pseudoglobulin giving practically the same figures which are smaller than those given by the native proteins (Table V).

But while the flocculating power of the native proteins can be inhibited by the presence of neutral salts no such effect can be detected with the filtrates obtained by denaturation by radiation, indicating a difference in the nature of the flocculation.

Table V. *Gold sol reactions. The figures indicate concentrations °/₀.*

	0·02	0·01	0·005	0·002	0·001	0·0005	0·00025
Serum-albumin	R_{vvv}	R_v	R	R	R	R	R
Pseudoglobulin	colourless	colourless	colourless	V	V	R_v	R
Filtrate from irradiated serum-albumin	colourless	colourless	colourless	V	R_{vvv}	R_{vv}	R_v

R = cherry red; V = violet; suffixes v = indicate shades of red with tinge of violet.

After dialysis and electrodialysis there is a nearly complete loss of the conductance, absorption and refractive power of the fluid which no longer gives the biuret reaction and shows only traces of its original gold sol-flocculating power.

Taking into consideration the properties of the filtrate here described it does not seem possible to identify the fraction or fractions with any known cleavage-product of proteins according to the classification of Pick [1925]. Nor does the gold sol reaction give any help. According to Zsigmondy [1920] both secondary proteoses and peptones are able to flocculate colloidal gold with blue colour without additional salt. Zunz [1906] has determinated in 13 different fractions of secondary proteoses and peptones the amount necessary to change the colour of red gold to violet. His most potent fraction (Albumose BI) has only 1/10 of the flocculating power of the filtrate obtained by irradiation. Zunz [1907] also stated that secondary proteoses are unable to flocculate mastic sol; the filtrate obtained by irradiation of proteins did precipitate mastic sol though in somewhat higher concentrations (Table VI).

Table VI. *Mastic sol reactions. The figures indicate concentrations °/₀.*

	0·086	0·043	0·021	0·01	0·005	0·002	0·001	0·0005	0·0002
Serum-albumin	×	×	×	×	+	0	0	0	0
Pseudoglobulin	×	×	×	×	×	×	+	0	0
Filtrate from irradiated pseudoglobulin	×	× +	+	0	0	0	0	0	0

(× = flocculation, + = cloudiness.)

It is only possible to state that the filtrate from irradiated protein is practically free from proteins and contains some secondary proteoses and probably peptones. But considering the very low acidity of the filtrate it seems scarcely likely that the flocculation of colloidal gold is caused by peptones.

In order to ascertain whether the special kinds of proteoses found in the filtrates of irradiated proteins are missing entirely from those of heat-coagulated proteins a 0·2 % solution of pseudoglobulin was heated on the water-bath for 15 minutes. After removing the precipitated protein the filtrate was concentrated to 1/50 vol. The solid content of such a solution amounted to 0·11–0·13 %. Reactions with sulphosalicylic acid and trichloroacetic acid were positive while the gold sol reaction remained entirely negative.

This seems to indicate that in denaturation of proteins by heat and by light of short wave-length, differences can be detected not only in the precipitated fraction [Spiegel-Adolf, 1927] but also in the cleavage products. Changes in odour and colour had already suggested this.

The fact that some proteoses show such a high degree of flocculating power towards colloidal gold seems to be of some interest in connection with the behaviour of spinal fluids in certain pathological cases. Though the results of the reaction of Lange can nearly always be explained by the content of proteins in the fluid and by the ratio between albumin and globulin, nevertheless there are

cases such as the one referred to by Kyrle *et al.* [1920] in which there was a marked gold sol flocculation without an increase in proteins. Besides, Epstein and Rubinstein [1926], using the adsorption method of Willstätter, were able to show that the fractions giving gold sol curves characteristic for general paralysis did not show protein reactions. Though no identity between this substance and the one obtained through irradiation can be reasonably expected, experiments were started in which an equal amount of the latter was added to a spinal fluid and the gold sol reaction of this mixture compared with that of the spinal fluid alone.

The spinal fluids were sterile, Wassermann-negative and did not show any increase in proteins. As in all previous tests two kinds of gold sol were used simultaneously, one made according to the method of Bredig with consecutive purification by dialysis, the other by reduction with formaldehyde. The precipitation was made according to Lange. The results of such an experiment are given in Table VII.

Table VII. *Spinal fluid "K.M." containing equal amounts of 40 times concentrated filtrate from irradiated protein.*

Diluted ...	10	20	40	80	160	320
Red	.	.	.	.	.	.
Red-violet	.	.	.	.	+	+
Violet	.	.	.	+	.	.
Blue	.	.	.	.	.	.
Bluish white	+	+	+	.	.	.
White	.	.	.	.	.	.

The spinal fluid alone when diluted in the same way did not change the colour of the red gold sol.

These experiments show that the addition of proteoses derived from horse-serum by irradiation elicits in normal spinal fluid a behaviour towards colloidal gold practically identical with the reaction of the same fluid in cases of general paralysis. The test of Nonne-Appelt was negative. Nevertheless there seems to exist a protective power in normal body fluids against the gold sol-precipitating effect of the proteoses, as a much greater amount of the latter was necessary to obtain effects similar to those reached with watery dilutions of the proteoses alone. Solutions of pure serum-albumin or pseudoglobulin did not show this effect.

The behaviour of the proteoses obtained by irradiation seems to suggest the occurrence of other atypical proteoses in spinal fluids in pathological cases as an explanation of a positive gold sol test without increased protein content.

Summary.

1. Protein solutions of 0·1–0·2 % irradiated or heated in the presence of acid or alkali show practically unchanged refraction.

2. Electrolyte-free serum-albumin and pseudoglobulin in concentrations from 0·2 to 0·002 % are practically entirely coagulable by heat or irradiation; in the latter case, only the filtrate shows an increase in refraction and a positive reaction with colloidal gold.

3. Protein solutions of 0·2–0·5 % containing acid or alkali do not show, after irradiation, neutralisation and heat-coagulation, positive reactions with colloidal gold.

4. Filtrates of protein coagulated by heating or irradiation were concentrated 10–50 times and investigated physico-chemically. The results seem to indicate the presence of atypical proteoses in the latter case.

5. Evidence is given that the proteoses are distinguished by strong light absorption. Their power to flocculate colloidal gold exceeds the power of genuine proteins 10–40 times. They also precipitate mastic sol.

When a concentrated solution of the proteoses obtained by irradiation of electrolyte-free proteins is added to normal spinal fluid the mixture gives a gold sol reaction similar to that obtained in cases of general paralysis.

The author wishes to express her thanks to Dr Temple Fay for kindly providing spinal fluids.

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